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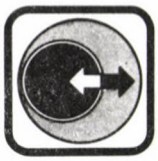

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EDITED BY

E. BÁCSY, G. B. MAKARA, GY. RAPPAY, P. RÖHLICH and D. SZABÓ

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International congresses on cell biology

First International Congress on Cell Biology
Boston, USA, September 5—10, 1976
Second International Congress on Cell Biology
Berlin-West, August 31—September 5, 1980
Third International Congress on Cell Biology
Tokyo, Japan, August 26—31, 1984

European congresses on cell biology

First European Congress on Cell Biology
Paris, France, July 18—23, 1982
Second European Congress on Cell Biology
Budapest, Hungary, July 6—11, 1986

GUIDE-LINE

Abstracts of contributions—both oral and poster presentations—to the Second European Congress of Cell Biology are collected in this issue of *Acta Biologica Hungarica*. A full-page abstract was offered to plenary lecturers and invited symposium speakers who have partly taken this opportunity.

All abstracts are arranged thematically and numbered consecutively. These numbers are referred to at each item of the programme booklet.

CONTENTS

1 Higher Order Structures of Chromatin and Nucleosome Positioning.

Fritz Thoma, Institut für Zellbiologie, Eidgenössische Technische Hochschule, CH-8093 Zürich.

The high DNA content of eukaryotic cells requires a dense packaging into nucleosomes and chromatin fibers, whereas transcription, replication and their regulation demand selective access to DNA sequences. Thus, removal of histones and altering positions of nucleosome with respect to the DNA sequences is one way to modulate gene expression. Chromatin also has non-nucleosomal regions such as the linker DNA between nucleosomes, nuclease sensitive sites or centromers which adopt different structures and might interfere with formation of regular fibers. We would like to know what determines the arrangement of nucleosomes along the DNA sequences, in particular with respect to genes and their regulatory regions. Several mechanisms for non-random placement of nucleosomes can be envisaged: (i) If nucleosomes were formed as soon as the appropriate length of DNA is replicated, the nucleosomes would line up with respect to the origin of replication. (ii) Sequence specific properties of DNA (like bending) might lead to specific protein (histone) - DNA interactions. (iii) Positioned nucleosomes might occur if the random arrangement is restricted by boundaries, such as nuclease sensitive regions or positioned non-histone proteins.

We have used the following strategy to study mechanisms of nucleosome positioning in vivo in yeast S. cerevisiae. The chromatin structure of a yeast plasmid (the TRP1ARS1 circle) was determined. Derivative plasmids were constructed by insertions of DNA fragments at points of interest and the alterations in chromatin structures were determined after reassembly into chromatin in vivo. The chromatin structure of the TRP1ARS1 circle consists of (i) four unstable nucleosomes on the TRP1 gene, (ii) one nuclease sensitive region around the 3' end of the gene and the putative origin of replication (ARS1), (iii) a second nuclease sensitive region at the 5' end of the gene and (iv) three stable and precisely positioned nucleosomes (I, II, III) on a sequence of unknown function (Thoma et al., J. Mol. Biol. (1984) 177, 715-733). Insertions of DNA between nucleosomes I and II resulted in long linkers and additional nucleosomes, but had no effect on the positions I, II and III. Therefore, replication or boundary conditions were unlikely to determine these particular positions. There seem to exist positioning signals on the DNA sequence which direct the formation of nucleosomes (Thoma & Simpson, Nature (1985), 315, 250-252). Insertion of the URA3 gene into the TRP1 gene resulted in changed nucleosome positions on the TRP1 gene. These changes correlate to changes in the flanking nuclease sensitive region, which demonstrates that nuclease sensitive regions can act as boundaries to determine nucleosome positions. Since on the same sequence (the TRP1 gene) nucleosomes were formed at different positions depending on the flanking structures only, histones might form nucleosomes at preferential positions as long as no other restrictions apply (Thoma (1986), J. Mol. Biol., in press). Putative regulatory sequences like the 'TATA' box of the URA3 gene or the ARS1 consensus sequence were found at the border of nuclease sensitive regions and precisely positioned nucleosomes. Therefore, a role of nucleosomes could be to present important sequences to the solution.

2 ORGANIZATION OF CHROMATIN AND NUCLEOSOMES OF ACTIVE AND INACTIVE GENES.

A.V.Belyavsky, S.G.Bavykin, K.K.Ebralidze, V.V.Shick, V.L.Karpov, O.V.Preobrazhenskaya, V.M.Studitsky and A.D.Mirzabekov. Institute of Molecular Biology, USSR Academy of Sciences, Moscow 117984, USSR

Using DNA-protein crosslinking, the following refined map of histone arrangement along one strand of DNA in core nucleosomes has been obtained:
$5'$- $H2B_{25,35}$ - $H4_{55,65}$ - $H3_{75,85,95}$/$H4_{88}$ - $H2B_{105,115}$ - $H2A_{118}$ - $H3_{135,145}$ - $H2A_{145}$ - $3'$
(only strong contacts are shown). Tetramer $(H3,H4)_2$ and dimers (H2A,H2B) are arranged on double-stranded core DNA symmetrically and relatively independently from each other:
H2A/H3 - (H2A,H2B) - $(H3,H4)_2$ - (H2A,H2B) - H3/H2A. Primary organization of core nucleosomes has been found to be highly conservative in organisms ranging from lily and yeast to mammals.

Primary organization of sea urchin sperm nucleosomes has been studied in some detail. Nucleosome core occupies several places spaced by 10 bp. in the center of nucleosomes containing full complement of histones and DNA of about 235 bp. in length. The crosslinking of histones H2B and H3 to spacer DNA in these nucleosomes has been detected, which suggests that these core histones, alongside with H1, are involved in the spacer organization. The data are consistent with the solenoidal model of chromatin in which folded 10-nm filament is formed by continously supercoiled core and spacer DNA.

Primary organization of nucleosomes depends to some extent on their conformational state. Thus, considerable enhancement of H4(55) contact over other H4 contacts was found for nucleosomes partially unfolded in low salt or urea, as well as for chromatin depleted of histone H1. Another feature is the appearance of H2A(75) contact after removal of histone H1 from chromatin.

A novel approach for mapping histone regions interacting with different sites of nucleosomal DNA has been developed on the basis of ^{32}P labeling of histones at the sites of crosslinking. For histone H4, the same set of peptides has been found to be crosslinked at (55), (65), and (85) binding sites. It is suggested that these peptides form one binding domain interacting with closely spaced nucleosome sites $\pm$ 1, $\pm$ 1.5, and $\pm$ 2 across the compressed DNA grooves.

Interaction of HMG 14 and HMG 17 proteins with nucleosomes was studied by protein-DNA crosslinking and immunoaffinity isolation. These proteins were shown to be crosslinked to DNA in core nucleosomes at four sites: at $3'$ and $5'$ DNA termini and at a distance of about 25 and 125 bases from $5'$ ends. The HMG binding sites (25) and (125) are arranged opposite each other across the minor groove on the inner side of nucleosome DNA superhelix. Binding of HMG 14/17 to nucleosomes does not induce any detectable rearrangement of histones on DNA.

Using "protein image" hybridization technique it was shown that histone content of a particular gene is correlated with its transcriptional activity. Silent hsp 70 genes of Drosophila contain full complement of histones. Moderate transcription of hsp 70 genes is accompanied by removal of histone H1, whereas high level of transcription during heat shock results in almost complete removal of all histones. It is suggested that the removal of histones is brought about by transcribing RNA polymerases, so that histone content should be dependent on the density of polymerase molecules on the gene and the rate of reconstitution of histone octamers with DNA. Promoter region of hsp 70 genes is free from histones in active as well inactive state, which seems to be necessary for rapid induction of the gene.

Hybridization of two-dimensional DNA-electrophoresis patterns of crosslinked nucleosomes from Drosophila embryos with probes derived from transcribed hsp 70 gene and non-transcribed ribosomal insertion type 1 has not revealed any substantial differences in the primary organization of nucleosomes on active and silent sequences. These findings are again consistent with the hypothesis of reversible removal of histone octamers by transcribing RNA polymerases.

3 NUCLEAR PROTEINS IMMUNOLOGICALLY RELATED TO TROUT HIGH MOBILITY GROUP PROTEIN T (HMG T) ARE LOCATED IN PUFFS OF CHIRONOMUS PALLIDIVITTATUS POLYTENE CHROMOSOMES. Hans-H. Trepte, Reiner Westermann*. III. Zoologisches Institut-Entwicklungsbiologie, Universität Göttingen, Berliner Str. 28, D-3400 Göttingen.*Present address: Institut für Anatomie und Zellbiologie, Universität Marburg, R.-Koch-Str. 6, D-3550 Marburg. Federal Republic of Germany

Trout HMG T protein is analogue to mammalian HMG 1 and 2. It is suggested that HMG T protein is associated with actively transcribed DNA. We have used an anti-trout HMG T-antibody to analyse the distribution of HMG T-related protein(s) in the salivary gland chromosomes of Chironomus pallidivittatus by means of indirect immunofluorescence. Squash preparations of the salivary gland cells which where incubated with anti-trout HMG T-antibodies, showed a very specific and strictly reproducible pattern of indirect immunofluorescence. The fluorescence was restricted to the puffs. The Balbiani rings (giant puffs) exhibited the most intensive fluorescence at the periphery. At chromosomal sites where prominent puffs occur, a bright fluorescence was regularly observed, whereas the fluorescence of the minor puffs was of a lower intensity. The fluorescence of the bands was negligibly low compared to that of the puffs. The nucleolus showed only very weak fluorescence. We conclude that all fluorescent sites were actively transcribing puffs and that all puffs exhibited indirect immunofluorescence. The results show that protein(s) immunologically related to trout HMG T are bound to chromatin of puffs in insect giant chromosomes. As we have already demonstrated, Chironomus proteins related to mammalian HMG 14 are also exclusively located in puffs. This shows that, in insects, proteins which are related to the main vertebrate HMG proteins seem to be restricted to transcriptionally active chromatin, suggesting that these insect proteins play a similar role. We thank G.H. Dixon for his gift of anti-trout HMG T-antibodies.

4 CHROMATIN STRUCTURE AND THE CELL- AND STAGE-SPECIFIC ACTIVATION OF THE LYSOZYME GENE.

Albrecht E. Sippel (1), Joachim Nowock (2), Manfred Theisen (1), Andreas Pueschel (1), Uwe Borgmeyer (1), Aribert Stief (1), Hans Fritton (3), Tibor Igo-Kemenes (3), Hartmut Beug (4), Thomas Graf (4).

(1) Zentrum für Molekulare Biologie, Universität Heidelberg, INF 282, D-6900 Heidelberg, FRG; (2) Heinrich Pette Institut, Martinistr. 52, D-2000 Hamburg, FRG; (3) Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Universität, Goethestr. 33, D-8000 München, FRG; and (4) European Molecular Biology Laboratories, Meyerhofstr. 1, D-6900 Heidelberg, FRG.

Structural studies on the chromatin domain of the chicken lysozyme gene led to the discovery of nine DNAase hypersensitive sites clustered around the gene. Alternative sets of these sites exist depending on whether the gene is constitutively expressed in mature macrophages, steroid controlled in oviduct cells, or inactive like in liver cells (1,2). In hematopoiesis, the lysozyme gene can be considered to be a marker gene for late myeloid differentiation. Distinct retrovirus transformed cells, representing various stages of chicken macrophage and erythrocyte precursor cells (3), were used for chromatin structural studies in the lysozyme gene region. We found that the formation of the active chromatin structure at the lysozyme enhancer region (4) must be an early event in myeloid determination and that the full activation of the lysozyme gene in late macrophage differentiation is correlated with chromatin structural changes at distinct sites halfway between enhancer (-6.1 kb) and promoter (0.1 kb).
In order to understand the molecular structure and function of the DNAase hypersensitive regions in chromatin, we used DNA sequences contained within nuclease hypersensitive regions (a) to construct recombinant plasmids for gene transfer experiments and (b) for in vitro DNA binding of nuclear proteins. The in vivo functional assays show that the far upstream enhancer element is responsible for the formation of the open chromatin structure at the promoter region and the transcriptional activation of the lysozyme gene (4). In vitro reconstitution of the enhancer DNA: protein structure revealed an unexpectedly complex element of many transregulatory protein factors. One of these DNA binding proteins is the chicken TGGCA protein (5), a functional homologue to HeLa cell nuclear factor I (6,7).

(1) Fritton et al. (1984) Nature 311, 163-165.
(2) Sippel et al. (1986) in Cancer Cells 4; DNA Tumor Viruses. Cold Spring Harbor Press, USA.
(3) Beug et al. (1979) Cell 18, 379-390.
(4) Theisen et al. (1986) EMBO J. 5, April.
(5) Borgmeyer et al (1984) Nucleic Acids Res. 12, 4295-4311.
(6) Nowock et al. (1985) Nucleic Acids Res. 13, 2045-2061.
(7) Leegwater et al. (1986) EMBO J. 5, 381-386.

5 EFFECTS OF NUCLEAR MATRIX PREPARATION ON LOCALIZATION OF NUCLEAR ANTIGENS IN 3T3 FIBROBLASTS AND BOVINE LYMPHOCYTES. N. Chaly(1), T. Bladon(1) and D.L. Brown(2), Departments of Biology, Carleton University(1) and University of Ottawa(2), Ottawa, Ontario, Canada.

The association of numerous nuclear functions with the nuclear matrix(NM) has consistently been questioned on the grounds that the harshness of the isolation protocol may be inducing artefactual associations, and serious doubts still exist as to whether the isolated NM, a putative nuclear skeleton, has a structural and functional counterpart in nuclei in situ. We have approached this problem by monitoring the localization of a variety of nuclear antigens at each stage of NM preparation. We have selected some non-histone antigens whose distribution in intact nuclei and NM has been previously characterized (lamins, centromeres) as well as some detected by monoclonal antibodies generated by immunizing mice with total NM (Chaly et al., J.C.B. 99, 661, 1984). NM of mouse 3T3 fibroblasts and of mitogen-stimulated bovine lymph node lymphocytes were prepared by processing cells through a modified Berezney and Coffey extraction protocol, and indirect immunofluorescence staining was performed at each stage of extraction. Similar results were obtained with both cell types. Nuclear antigens were (1) unaffected (e.g. lamins, PI1), (2) partly extracted but not redistributed (e.g. centromere antigens, PI2), (3) not extracted but redistributed upon removal of chromatin (e.g. P1, perichromin) or (4) extracted and/or redistributed at each stage of NM preparation. From these observations we conclude that the organization, and hence the function, of some components in isolated NM (sets 1 & 2) reflect their organization in situ. Antigens in set 3 may play a role in the spatial ordering of chromatin, perhaps by interaction with the lamins and other yet unidentified nuclear factors. It is clear, however, that some nuclear components are only fortuitously associated with the NM (set 4), indicating the need for caution when evaluating the role of NM-associated polypeptides in nuclear organization. (Supported by Medical Research Council of Canada).

6 EVIDENCE OF REPETITIVE THREE-DIMENSIONAL PATTERNS OF CHROMATIN DISPOSITION IN THE INTERPHASE NUCLEUS OF RAT LIVER CELLS IN SITU. Clara Esquivel, Olga Echeverría, Gerardo Vázquez-Nin. Laboratory of Electron Microscopy, Faculty of Sciences, UNAM, México.

It is well known that chromatin is not distributed at random inside the interphase nucleus. However,its detailed disposition is almost unknown. In the present work the distribution of compact chromatin was studied by means of 107nm thick serial sections for electron microscopy. Preferential contrast of chromatin was achieved using block staining with phosphotungstic acid at pH 2.3. Models of the chromatin of whole nuclei were made at 29000 X. All grains larger than 138nm and all filaments thicker than 34nm were presented. At this resolution chromatin looked like a three-dimensional network of sheets, threads and aggregates in which no isolated fragment can be distinguished. Nevertheless, some outstanding structures as large bodies and clusters of elements could be recognized in different nuclei. Models were rotated in order to superimpose the nucleoli, and then outstanding structures recognized in the central part of the nucleus were compared. Coincidences were constantly found in several nuclei allowing us to configure a three-dimensional pattern of similitudes involving most of the chromatin of each nucleus. These initial qualitative comparisons will be continued by computer aided studies of interphase and mitotic nuclei,in an effort to know more about the order of the arrangement of chromosomes during the interphase.

7 ION-DEPENDENT CONFORMATIONAL TRANSITIONS AS INDICATORS OF SPECIFICITY OF TOTAL CHROMATIN IN DIFFERENT TYPES OF CELLS. H.T. Khachatrian, G.G.Karaghebakian, A.A. Matnishian, Yu.A. Magakian. Institute of Experimental Biology of the Academy of Sciences of the Armenian SSR, 7, Hasratian str., Yerevan, 375044, USSR.

Specificity of the structure of total chromatin was studied using digestion with pancreatic DNAase (DNAase I) in situ. In the presence of 3 mM $MgCl_2$ and no monovalent cations chromatin of avian erythroid cells is cleaved at ~400 bp intervals ("double-nucleosome" periodicity). Chromatins of rat liver, thymus, spleen and kidney cells are cleaved at 200 bp intervals but the fragments with lengths which are multiples of 400 bp predominate ("400+200 bp" mode of fragmentation). The mode of chromatin fragmentation in situ correlates with the state of the condensed chromatin in the nuclei. Thus, in the presence of 0.5 mM $MgCl_2$, 200 mM NaCl, rat liver chromatin displays a "400+200 bp" mode of fragmentation. Lowering NaCl concentration to the value of 20 mM, which causes dispersal of condensed chromatin in situ (Dixon, Burkholder, Eur. J. Cell Biol., 36, 315, 1985), leads to cleavage of chromatin at 100 bp intervals. In all types of cells studied a similar in situ transition of the chromatin structure is observed due to lowering magnesium concentration in solutions lacking sodium ions. In chicken erythrocyte nuclei transition to the 100 bp mode of fragmentation takes place at ~0.75 mM $MgCl_2$, in nuclei of chicken non-anemic polychromatophilic erythroblasts and of rat thymocytes - at ~1 mM $MgCl_2$, in rat liver nuclei - between 1.5 and 1.25 mM. Thus, this magnesium-dependent structural transition of chromatin possess limited cell-type specificity.

8 ULTRASTRUCTURAL CHANGES OF RIBOSOMAL CHROMATIN INDUCED BY COUMERMYCIN INHIBITION OF ATP-DEPENDENT DNA TOPOISOMERASE IN TG-CELL NUCLEOLI. M.Derenzini, Fulvia Farabegoli, Annalisa Pession, F.Novello. Istituto di Patologia Generale, Via San Giacomo 14, 40126 Bologna, Italy.

We have studied by electron microscopy the effects of coumermycin, an inhibitor of the ATP - dependent DNA topoisomerase, on the structure of ribosomal chromatin of TG cells in-situ. During interphase ribosomal chromatin is located in the fibrillar components of the nucleolus, the fibrillar centers and the associated dense fibrillar component. This chromatin, as revealed by the osmium-ammine staining for DNA, is structured in loose agglomerates composed of completely extended DNA filaments, 2-3nm thick, which never give rise to nucleosome-like structures. In untreated interphase TG-cells nucleolar ATPase activity, cytochemically detected, was located in the fibrillar centers and the dense fibrillar component. As early as 45 min after coumermycin treatment at the dose of 50ug/ml, the cytochemical reaction for nucleolar ATPase became negative. At the same time, the osmium-ammine staining for DNA, revealed that the agglomerates of ribosomal chromatin were characterized by the presence of rare filaments with a thickness of 6-7nm. Even after coumermycin treatment, nucleosome-like structures were not visualized in the ribosomal chromatin.

9 CHANGE IN THE CHROMATIN STRUCTURE INDUCED BY INTERACTION OF 2N-METHYL-9-HYDROXY ELLIPTICINIUM.

Lionel LARUE and Jacques PAOLETTI Unité de Biochimie, Institut Gustave Roussy, 94805 Villejuif, FRANCE.

NMHE : 2N-Methyl-9-Hydroxy Ellipticinium (CELIPTIUM) possess antitumor activity, binds to DNA by intercalation (Kap = 10^6 M^{-1}), inhibits cellular DNA and RNA synthesis more efficiently than protein synthesis, and is able to bind topoisomerase II. We study the effects of NMHE on the structure of chromatin, using micrococcal nuclease and DNase I as structural probes.
(1) The interaction of NMHE with naked-DNA inhibits the digestion of the DNA. (2) The interaction of NMHE with isolated chromatin or with chromatin into isolated nuclei induces two structural changes of chromatin. (a) An unfolding of the overall structure which causes an activation of the rate of degradation by the nucleases and (b) an unwrapping of the DNA from the histone core leading to a disorganisation of the core particle structure.(3) The binding of NMHE to the genome in isolated nuclei induces an inhibition of the digestion of the DNase I sensible regions indicating that these regions are more accessible to the drug than the inactive regions.

10 PERICENTROMERIC HETEROCHROMATIN: ISOLATION AND PARTIAL CHARACTERIZATION

Elena Stephanova (1), Valya Russanova (2). (1) Faculty of Biology, University of Sofia, 1421 Bulgaria, and (2) Institute of Molecular Biology, Bulgarian Academy of Sciences, Sofia 1113, Bulgaria

A method is suggested for a selective condensation of pericentromeric heterochromatin by treating mouse liver nuclei with decreasing concentration of divalent cations. In situ hybridisation with labeled mouse satellite DNA shows that the radioactivity was entirely associated with these structures. The latter were isolated and their DNA and protein components analysed. The pericentromeric heterochromatin is almost devoid of nonhistone proteins, acetylated forms of histones and protein A_{24}. The quantitative ratio of non-allelic variants of histons H_{2A}, H_{2B} and H_3 also is different as compared to the bulk chromatin.

11 ULTRASTRUCTURE AND ELECTROPHORETIC PROTEIN PATTERN OF A NUCLEAR FRACTION ENRICHED IN INTERCHROMATIN GRANULE CONGLOMERATIONS.

S.Krzyżowska-Gruca, A.Zborek, S.Gruca. Department of Tumor Biology, Center of Oncology, 44-100 Gliwice, Poland.

Rats were injected with a cytostatic 1-nitro-9/3´-dimethylpropyloamine/acridine.2HCl to induce aggregation of interchromatin granules /IG/. The conglomerations of IG were well preserved in isolated liver nuclei and in nuclear structures deprived of chromatin. This feature enabled obtaining a nuclear fraction enriched in IG. Briefly, the method consisted in extraction of isolated nuclei with a non-ionic detergent and digestion with DNase I in a high ionic strength. Each step of isolation was ultrastructurally monitored using both the routine electron microscopy as well as a preferential staining of IG with bismuth. Presence of spots of tightly packed granules within IG conglomerations in the final fraction like in the nuclei in situ was a good ultrastructural marker of IG. The resulting fraction consisted predominantly of IG conglomerations. Their preferential staining with bismuth was well preserved. Minute amounts of fibrillar material originating from nuclear matrix and residual nucleoli could be observed. Protein composition of the fraction enriched in IG was studied by SDS-polyacrylamide gel electrophoresis. After electrotransfer, nitrocellulose filters were fixed with glutaraldehyde and stained with bismuth method in order to identify IG proteins. The results of ultrastructural and cytochemical studies in comparison to electrophoretic protein pattern are discussed.

12 THE ROLE OF GENETIC BALANCE IN CONTROL OF X CHROMOSOMAL ORGANISATION FOR DOSAGE COMPENSATION IN DROSOPHILA MELANOGASTER. R. N. Chatterjee. Department of Zoology, University of Calcutta, 35 Ballygunge Circular Road, Calcutta 700019, India

The chromatin template activity of the polytene X and autosomes of larval salivary glands of Drosophila melanogaster has been assayed as a function of the ratio of X chromosome to the set of autosomes (X : A). Autoradiographic analysis of ^{3}H-UMP labelling under in situ assay condition upon the use of exogenous E. coli RNA polymerase (holoenzyme) revealed that, if the X : A ratio was 1 or more (XX : AA or XXX : AA), the template activity of the X chromosome was set at a 'female level' and if 0.5 (1X : AA) X activity was at a 'male level' (twice as high as individual X chromosome of female). However, in aneuploid males with normal X plus a proximal piece of X fragments (X + X fragments, AA) of different size, the X chromosomal template activity varies from one group of hyperploids to another and this variation could not in every case be explained by X : A dosage ratio or 'sex index'. In the male, a duplication of a proximal part of the X has been found to reduce the template activity of the distal part. On the other hand, trisomy for the entire left arm of either the chromosome 2 (2L) or chromosome 3 (3L) had no recognisable influence on X chromosomal organisation, although these individuals have a 'sex index' below 1 (0.89) in the case of the female and below 0.5 (0.45) in case of the male. These results have been interpreted to have suggested that the template activity of the X chromosome does depend on the 'sex index', only in a threshold wise manner.

13 FLUORESCENCE PATTERNS OF MITOTIC AND POLYTENE CHROMOSOMES OF THE GENUS BOETTCHERISCA (SARCOPHAGIDAE: DIPTERA). R.R.Tewari (1,3), Hiromu Kurahashi (2) and Vandana Rai (3). (1) Department of Medical Zoology (Chief: Prof. Rokuro Kano), Tokyo Medical and Dental University, 5-45, Yushima, Bunkyo-ku, Tokyo 113, Japan, (2) The National Institute of Health, 10-35, Kamiosaki, Shinagawa-ku, Tokyo 141, Japan, (3) Department of Zoology, University of Allahabad, Allahabad-211002, India.

A study of the patterns of fluorescence both in mitotic and polytene chromosomes in eight species of flesh-flies of the genus Boettcherisca, namely B. koimani, B. karnyi, B. peregrina, B. nathani, B. javanica, B. timorensis, B. septentrionalis and B. invaria was carried out using the fluorochromes Hoechst 33258 and DAPI.

All the species possess similar mitotic karyotypes with five pairs of meta/submetacentric autosomes which are represented in the polytene nuclei of the pupal foot pad cells as banded elements - and a pair of small dot like sex chromosomes, XX in the females and XY in the males. The sex chromosomes do not polytenize in any of the species, however, in some species they are represented in the form of heterochromatic granules. Consistent differences in the patterns of fluorescence in both the polytene and the mitotic chromosomes are found between different species. These findings have been correlated with the taxonomic groupings of these species.

14 EVOLUTION OF SEX-CHROMOSOMES IN LACERTID LIZARDS. E.Olmo, T.Capriglione, O. Cobror and G.Odierna. Dipartimento di Biologia Evolutiva e Comparata, via Mezzocannone 8, 80134, Napoli, Italy.

Singh and co-workers (1976, 1980) have suggested that in snakes sex-chromosome differentiation starts with the segregation on either homolog of a specific satellite DNA, accompanied by heterochromatinization. This may subsequently be followed by structural rearrangement of the heterochromatinized homolog.

A study on the sex-chromosomes of various lacertid lizards seem to show that in these reptiles the sex-chromosome differentiation may go through the same evolutionary steps as in snakes. In fact in the females of Takydromus and Gallotia, two of the most primitive lacertids, the two sex-chromosomes have the same size and shape, but the W-chromosome differs from the Z in being completely heterochromatic and C-banding positive. It probably contains a DNA enriched in AT, because it is strongly positive to the fluorochrome DAPI stain. In most of the more evolved species the W-chromosome is still completely heterochromatic, but also smaller in size. It is hypothesized that this W-chromosome evolved through a deletion mechanism from a W-chromosome similar to that of Takydromus and Gallotia.

A biochemical research is presently in progress to verify the presence in the W-chromosome of lizards of a sex-specific DNA similar to that found in snakes.

15 Electron microscopic immunolocalization of a 80 kD nuclear antigen in the oocytes and somatic cells of the amphibian Pleurodeles waltlii.

Chandra K. PYNE, Laboratoire de Génétique du Développement, Université P. et M. Curie, 9 Quai St. Bernard, 75005 Paris, France.

A library of monoclonal antibodies directed against proteins of oocyte nuclei of P. waltlii has been started in this laboratory. One monoclonal antibody, A33/22, recognizing a 80 kD protein in immunoblots, labels all the loops of lampbrush chromosomes as well as the nucleoplasm of the oocytes in all stages of devlopment; the nucleoli are not labeled (1). In an earlier electron microscopic study of liver cells, using indirect immunoperoxidase labeling before embedment, we found a strong labeling of fibrillar structures in the interchromatin space; condensed chromatin, nuclear envelope and nucleoli were not found to be labeled(2).

For the present study, we have used the immunogold labeling procedure on thin sections of various tissues, embeded in Lowicryl K4M after different fixation procedures. In the oocytes, the immunogold labeling was distributed all over the nucleoplasm, with a preferential labeling of structures representing sections of chromosomal loops. Nucleoli and some other dense structures were not labeled. In the follicular and epithelial cells of the ovary, liver and thymus cells, the immunogold labeling was restricted to fibrillar structures in the interchromatin space, while the condensed chromatin and nucleoli were not labeled. These results are confirmed by immunoperoxidase labeling of these somatic cells.

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16 HEAT SHOCK PROTEINS OF MAMMALIAN NUCLEAR MATRIX

S.B. Akopov, T.V. Buldyaeva, S.N. Kuzmina, I.B. Zbarsky. N.K. Koltzov Institute of Developmental Biology, USSR Academy of Sciences, 117334 Moscow, Vavilov Street 26, U.S.S.R.

Heating of cultured Chinese hamster fibroblasts at 46°C for 10 min with subsequent maintenance at 37°C strongly inhibited the incorporation of ^{35}S methionine during first 4 hours into whole cell proteins and only slightly into proteins of the nuclear matrix. In 6 - 8 hours after heating several heat shock proteins in region of 120 - 150 kDa, about 80, 70, and 27 - 22 kDa revealed in fluorograms of ^{35}S methionine labelled proteins separated in SDS-polyacrylamide gel. Heat shock proteins p70 and three bands between 120 and 150 kDa were much more prominent in the nuclear matrix than in whole cell nuclei or whole cell proteins.

Heating of rats in vivo at 45°C and 22% humidity for 2 hours decreased the incorporation of labelled amino acids (^{14}C Chlorella protein hydrolysate) into liver whole cell and nuclear proteins but increased by 30% the incorporation into nuclear matrix proteins. Some alteration in protein profile of the nuclear matrix, namely a decrease of characteristic peaks of lamins and an increase in peaks of 100, 55, 40, and 30 kDa was also observed.

17 A COMPARATIVE STUDY OF SOME TIGHTLY BOUND PROTEINS FROM THE SPERM CHROMATIN OF TWO EVOLUTIONARY DISTANT SPECIES.

Z. Avramova, B. Tasheva. Institute of Molecular Biology, Bulgarian Academy of Sciences. Sofia 1113, Bulgaria.

The tightly bound nonprotamine proteins (those resisting extraction with 2M NaCl) of two sperm chromatins (ram and mussel) were compared by both two dimensional (2D) tryptic peptide map analysis and by one dimensional (1D) partial hydrolysis mapping. The tightly bound proteins from the sperm chromatin of the mammal were found different from those of the mussel when compared by 2D tryptic peptide mapping. Their 1D partial digestion profiles, however, suggested some relation.

An interesting observation was the similarity found among the proteins within each sperm chromatin type. Results similar to these have been reported for a subfamily of cytokeratin polypeptides which were found to be closely related to each other on the basis of their 2D tryptic peptide maps irrespective of considerable differences in their molecular weight. It may be speculated that common principles, i.e. expression of multigenic families, specific proteolytic cleavage or others, might be responsible for the origin and diversity of the cellular skeletal proteins.

18 ON THE FUNCTION OF THE NUCLEAR LAMINA

Ricardo Benavente, Georg Krohne and Werner W. Franke. Institute of Cell and Tumor Biology, German Cancer Research Center, 6900 Heidelberg, Federal Republic of Germany

The nuclear lamina is a proteinaceous fibrillar meshwork located subjacent to the inner nuclear membrane. This karyoskeletal structure is transiently depolymerized into soluble subunits upon disintegration of the nuclear envelope during mitosis and meiosis. The function of the nuclear lamina is still unknown; however, it has been postulated that it provides an architectural framework for the organization of the nuclear envelope and the chromatin. To test this hypothesis we microinjected lamin antibodies (monoclonal and polyclonal) into PtK_2 cells during metaphase. We expected that antibodies will bind lamins in their soluble state, interferring with their repolymerization during telophase. For controls, we microinjected cells with antibodies showing diverse specificities (monoclonal antibody Lo46F7 specific for Xenopus lamins, a monoclonal DNA antibody, and commercial "second antibodies" coupled to FITC or rhodamine). Antibodies which do not bind to antigens in PtK_2 cells (Lo46F7, "second antbodies") were excluded from the nuclei of daugther cells, whereas the DNA antibody was localized inside nuclei. In control experiments, microinjection, per se, did not interfere with nuclear reformation, chromosome decondensation and cytokinesis. However, microinjection of antibodies specific for PtK_2-lamins significantly prolonged mitosis and induced severe structural alterations in reforming daugther nuclei.

19 AUTOIMMUNE RESPONSE DIRECTED AGAINST B LAMIN IN A PATIENT WITH THROMBOPENIA. M.N. Guilly (1) F. Danon (2), M. Bornens (1), J.C. Courvalin (1). (1) C.G.M. - C.N.R.S.- 91190 GIF-SUR-YVETTE (France) (2) INSERM (U 108) Hôpital St Louis, Paris (France).

Sera of patients with autoimmune diseases have been useful for the identification of nuclear proteins (1). By this approach, Mc Keon et al (2) have characterized the immunoglobulins of a patient (LS-1) with linear scleroderma as directed against the A and C nuclear lamins in mammalian cells. We have analyzed the serum (F) of a patient suffering from a severe thrombopenia which exhibits the same cytological specificity. Immunoblotting and immunoprecipitation experiments performed with HeLa nuclear proteins showed that the serum was specific for lamin B. This serum together with the LS-1 serum were used to study the respective amount of the three lamins in different cell types. This was performed by immunoblotting and immunoprecipitation of nuclear fractions enriched in lamina proteins. The main result was the virtual absence of A and C lamins in rat thymocytes, in the human T-lymphoblastic cell line KE 37 and, to a lesser extent in clones of normal human T cells. By contrast, the signal obtained for B lamin in these cells was similar to that obtained in other cell lines.

These results suggest either a cell-type-specific expression of lamins in mammals as it has been described for Xenopus laevis development (3, 4) or a cell-type specific proteolysis of these structural proteins.

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20 NUCLEAR-RINGS:AN ORGANELLES IN SEARCH OF A FUNCTION.Juan Aréchaga(1)and Gunter F.Bahr(2) (1)University of Granada.Spain and (2)Armed Forces Institute of Pathology.Washington,DC.USA.

Nuclear-rings (NR) are structures of recent description in the nuclear compartment (1,2)They are perfect torical elements attached to the inner surface of the karyotheca,apparently by a strong bonding mechanism.Nuclear membranes seem to be continous at the level of the NR,so permanent or anatomical holes through both nuclear membranes ("nuclear-pores") do not apper to exit.In addition, two relationships with chromatin fibers and cytoskeletal filaments were seen especially in cultured and phytohemaglutinin-treated cells.A constant number of six 250 Å chromatin fibers were found per NR and pseudorings of cytoskeletal filaments (maybe actin microfilaments) anchorings were observed on the outer surface of nuclear envelope.Two preparatory techniques were essential to understand the anatomy and relatioships of the NR:(a)spreadings of whole nuclei on an air-water interphase followed by critical point drying and (b)freeze-fracture in solid alcohols.Moreover,we used densitometric procedures and computer-assisted image processing.Morphometric determinations in human lim phocytes showed that the internal diameter of the torus is very constant (aprox.278 Å) while there are strong variations in other NR diameters.The statistical mean of NR volume was $1_8 72 \times 10^{-4_3}$ μ ,of dry mass was 6.2×10^{-16} g. and of the combined molecular weight of single NR was 3.7×10^{8} (estimated by a photometric procedure).All these data can help in the isolation of NR in enough amount for biochemical studies;however,the functional role of these novel organelles is enigmatic,although their position in the nucleocytoplasmic interphase could be a very important fact. (1)J.Cell.Biochem.Supp. 9a:5(1985),(2)"Nuclear Envelope and RNA Maturation"(Smuckler and Clawson Eds.)pp.23-50.Alan R.Liss New York.1985.(Supported by CAICYT grant 2057-83)

21 NUCLEOLAR MODIFICATIONS DURING OOGENESIS IN RAT OOCYTES. Nadine Antoine, Guy Goessens, Edoard Baeckeland. Institute of Histology and Embryology, University of Liège, B-4020 Liège, Belgium.

Various nucleolar components are observed in mammalian follicular oocytes but it is difficult to establish analogies with the classical nucleolar components of somatic cells. In fact, nucleolar ultrastructure changes during the follicular growth. The nucleolus which originally has a reticulated fibrillo-granular structure becomes entirely compact. These morphological nucleolar changes are accompanied by significant modifications in nucleolar transcriptional activity. It is well known that RNA syntheses gradually increase during the oocyte growth and that the process of nucleolar compaction in mature follicle is accompanied by an important decrease in RNA syntheses.

We have followed the ultrastructural evolution of the nucleoli during the follicular growth by means of cytological and cytochemical methods. The oocyte nucleolus in the primordial or primary follicle exhibits a reticulated structure and consists of strands of dense fibrillar component and aggregates of granular component. Small fibrillar centres are also recognized. After silver staining, numerous silver grains are deposited on the dense fibrillar component while fibrillar centres appear to have less stainability. The aggregates of granular component are mostly devoid of silver grains. Similar structural components are seen in secondary follicle nucleoli. Oocyte nucleoli from antral follicles are essentially made of conspicuous and homogeneous compact areas which are not positive after silver staining. At this stage, the homogeneous compact nucleolar areas often exhibit a central vacuole containing some nucleolar components. In order to define the nature and the functions of these homogeneous areas, cytochemical methods allowing detection of nucleic acids, proteins or lipids were performed at the photonic or at the ultrastructural level. The results obtained suggest that these nucleolar areas are essentially composed of proteins but not argyrophilic proteins. Further ultrastructural cytochemical investigations will be necessary in order to precise the exact nature and functions of this enigmatic nucleolar component.

22 FURTHER STUDIES ON SATELLITE NUCLEOLI. Karel Smetana(1), Zbyněk Likovský(2), Harris Busch(3). (1) Institute of Hematology and Blood Transfusion, Prague, CSSR, (2)Institute of Experimental Medicine, Prague, CSSR, (3)Baylor College of Medicine, Houston, USA

According to previous studies, satellite nucleoli represent solitary nucleolus organizer regions (NORs) which did not fuse and participate in the formation of characteristic nucleoli. The visualization of satellite nucleoli was facilitated by the introduction of cytochemical and immunofluorescence procedures for the demonstration of characteristic nucleolar proteins.

The number of cells containing satellite nucleoli apparently depends on the state of the nucleolar RNA synthesis. The number of hepatocytes containing satellite nucleoli decreased adter the experimental inhibition of nucleolar RNA synthesis with actinomycin D. However, the decreased number of satellite nucleoli was also noted in stimulated hepatocytes as well as rapidly proliferating malignant hepatocytes. The decreased number of stimulated or malignant hepatocytes with satellite nucleoli may be possibly produced to the fusion of these nucleoli with characteristic nucleoli. Similar decrease of the number of cells with satellite nucleoli was also observed after stimulation of resting lymphocytes.

23 NUCLEAR GLYCOGEN ACCUMULATION IN DIABETIC KIDNEYS.

Palle Holck, Ruth Rasch. Department of Cell Biology at the Institute of Anatomy. University of Aarhus, DK-8000 Aarhus C, Denmark.

Pronounced cytoplasmatic accumulation of glycogen is a well-known histological finding in untreated diabetes (Armanni, Erhlich, Ritchie, Rasch). The abnormal cytoplasmatic glycogen is widely distributed in the kidney cortex and outer stripe of outer medulla and has been located to distal parts of the nephrons.

Recently we found glycogen not only to be accumulated in the cytoplasm but also, even though infrequent, in nuclei. This nuclear phenomenon has not been described in diabetic kidneys before and it has not been recorded in experimentally diabetic animals.

Female Wistar rats with a body weight of 110 g were made diabetic by intravenous injection of 90 mg streptozotocin/kg body weight. During the experimental period the animals had blood glucose values about 17 mmol/l, constant glucosuria but no ketonuria. After 50 days a retrograde perfusion fixation of the kidneys was performed and tissue blocks were cut to contain the papilla as well as representative parts of all other kidney zones. The tissue was postfixated in osmium tetroxide, dehydrated in alcohol, embedded in Epon and 3 µm serial sections were cut from cortex to the papilla. The sections were stained with PAS and toluidine blue. Light microscopical tracing of single tubules revealed no PAS-positive material in the proximal nephron but in the distal nephron the cytoplasmatic staining was pronounced. However, PAS-positive staining of nuclei was discovered in the initial part of the thick ascending limb within a narrow field of the inner stripe of outer medulla. The abnormal nuclei appeared round with a narrow normal circumference but brightly red centrally. Electron microscopy showed that the PAS-positive nuclei were about normal size. The centers were filled with granules of about 30 nm and surrounded by a halo of normal nuclear structure. The abnormal nuclear content was interpretated as monoparticular glycogen granules due to the PAS-positive staining, the size of the granules and finally because it was digestable by amylase. Nuclear accumulations have been described before in the diabetic liver and here suggested to be due to hyperfunction. The significanse of the present findings in the kidney, however, remains to be investigated.

24 EM CYTOCHEMISTRY AND RELATIONSHIPS OF THE NUCLEAR BODIES FROM RAT UTERINE EPITHELIAL CELLS.

A. J. Cidadão. Department of Cell Biology, Gulbenkian Institute of Science, Oeiras, Portugal.

To investigate nuclear body (NB) origin and functions, I analyzed their composition and relationships, and compared the constitution of NB, nucleolus, and interchromatin granules (IG). Nafoxidine-injected immature female rats were used to increase NB frequency (1) , and the partial chromatin decondensation method (2) to obtain a better visualization of their relationships. Osmium-ammine was employed to demonstrate DNA (3) , and a RNase gold complex (4) /EDTA regressive technique (3) to visualize RNA/RNPs. The presence of basic (ethanolic PTA; 3) , Ag-NOR (5) , and phosphorylated (bismuth tartarate; 3) proteins, was also investigated. Periodic acid(PA)- osmium-ammine (6) , PA-alkaline bismuth (7) , and a Con-A ferritin conjugate were used to demonstrate sugar residues. Structures showing positive reactions for both proteins and sugar residues were considered as being formed by glycoproteins.

Simple NB (SNB) and the capsule of complex NB (CNB) are formed by basic non-phosphorylated proteins. The CNB core is generally composed of RNPs, but (rarely) it can also contain chromatin. Although basic and non-phosphorylated, core proteins are glycoproteins, and thus, different from those of SNB and CNB capsule. External DNA/RNA positive filaments are linked to SNB, and to the CNB capsule. Core RNP filaments attach to the capsule, or are in continuity with external RNPs. IG are frequently observed near NB, and both are the most resistant nuclear structures to EDTA bleaching.

SNB are different from fibrillar centres. It is unlikely that NB are hormone receptors, and most probably they are not involved in RNA transcription. Results support: the hypothesis of NB intertransformation, probably through the entrance/ elimination of RNPs; the nucleolar origin of the type IV CNB core; the formation of the core of types II and III CNB from the incorporation of IG; a possible role of NB in the spatial organization of chromatin.

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25 **PREFERENTIAL INHIBITION OF NASCENT REPLICON MATURATION CAUSED BY A PARTIAL DEPLETION OF NUCLEOTIDE POOLS.**

Krimer, D.B. and J.B. Schvartzman. Centro de Investigaciones Biológicas (CSIC). Velázquez 144, Madrid 28006, SPAIN.

In higher eukaryotes, replication occurs at thousands of small units called **"eukaryotic replicons"**. In each of these replicons, DNA synthesis begins at a replication origin and progresses bidirectionally. Replication stops whenever two forks that are growing in opposite directions meet. Once all the replicons had completed their replication, there is still the necessity to join nascent replicon-sized fragments. This process which involves both filling of the termination gap and ligation of the DNA backbone is called **"nascent replicon maturation"**. Ligation of early, middle, and late-S replicated replicon-sized molecules occurs synchronously during late-S and G2. The reason for this delayed maturation of nascent replicons remains a mistery, although it seems to be regulated via nucleotide pool size. We have investigated the effects of a partial depletion of nucleotide pools on nascent replicon maturation in root tips of Pisum sativum. For this purpose we labeled nascent DNA with radioactive precursors in the presence of sub-optimal concentrations of Hydroxyurea and 5-Aminouracil, and used autoradiography and alkaline sucrose gradients to determine the labeling index and the growing pattern of nascent DNA molecules. When root tips were treated with the inhibitors for approximately one cycle time, cells accumulated in late-S and G2, and during recovey they entered mitosis synchronously. Under these conditions, the time needed for nascent molecules to achieve replicon size was similar to the controls. On the contrary, the growing rate of replicon-sized molecules to achieve chromosomal size was dramatically slowed down. This observation strongly suggests that ligation of completed nascent replicons is more sensitive than synthesis and ligation of Okazaki fragments, to a partial depletion of nucleotide pools. Accordingly, we postulate that the cell synchronization induced with these drugs, could be simply due to the preferential inhibition of nascent replicon maturation caused by a partial depletion of nucleotide pools.

26 TIME DEPENDENCE OF BIOCHEMICAL PARAMETERS DURING INDUCTION OF DNA REPAIR.

I. Vincze/1/, Larisza Gueth/1/, P. Weniger/2/, Veronika Karcagi/1/, Judit Püspöky/1/, H. Altmann/2/. /1/ Department of Biochemistry, National Institute of Hygiene, Budapest, Gyáli ut 2-6., H-1097 Hungary, and /2/ Institute of Biology, Research Centre Seibersdorf, A-2444 Seibersdorf, Austria

After in vivo genotoxic events several biochemical functions go through changes in cellular systems - e.g. spleen lymphocytes - of experimental animals. One of these responses is the change of repair capacity of DNA damages induced by UV light irradiation. In the present work we have investigated the possible relations between poly/adenoribosyl/synthetase /PAR/ activity, UV endonuclease activity and alteration of the composition of dNTP pool. PAR synthetase activity changed parallel to induced DNA repair activity resulting in a correlation coefficient of $R=0.82$ /$P < 0.01$/ between the two parameters. There was no significant relationship between the change of endonuclease activity and induction of DNA repair /$R=0.45$; $P > 0.05$/. Induction of DNA repair by methyl methanesulfonate was different in the function of deoxypurine and deoxypyrimidine pools, respectively. Change of DNA repair described saturation curves in the function of dATP and dGTP concentrations of spleen lymphocytes /$R=0.75$; $P < 0.01$; and $R=0.72$; $P < 0.01$/, and produced cubic curves in the function of dTTP and dCTP content /$R=0.82$; $P < 0.01$; and $R=0.67$; $P < 0.02$, respectively/.

27 DNA REPLICATION DURING LIVER REGENERATION. EFFECTS OF ETHANOL. GEORGE G. SKOUTERIS, DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF OXFORD, SOUTH PARKS ROAD, OXFORD, OX1 3QU, U.K.

The effects of chronic ethanol feeding on hepatic regeneration were studied in rats after partial hepatectomy. DNA synthesis was used as the index of liver regeneration and estimated by measuring the incorporation of ^{3}H-thymidine into DNA and by autoradiography of labelled hepatocyte nuclei. The activity of some key enzymes for DNA synthesis were also determined. These were: thymidine kinase, deoxyadenosine kinase, deoxycytidine kinase, ribonucleoside reductase, nucleoside diphosphokinase, and DNA polymerase, both α and β.

Chronic ethanol feeding resulted in a significant reduction of liver cell DNA replication after partial hepatectomy. The levels of thymidine kinase, deoxyadenosine kinase, deoxycytidine kinase, nucleoside diphosphokinase and DNA polymerase were lower when compared with those of rats feeding on a normal diet.

When the rats feeding on an alcohol-containing diet were switched to a normal diet for 1 week before partial hepatectomy, the inhibition of the liver regeneration was absent. Thymidine kinase, deoxyadenosine kinase, deoxycytidine kinase, and ribonucleoside reductase levels were also restored to normal values following partial hepatectomy. DNA polymerase β was also slightly increased which could possibly be due to an increased repair activity of the liver cells.

This data suggests that after chronic ethanol feeding the liver regeneration is significantly delayed. We suggest that the inhibiting effect of ethanol is reversible.

28 INDUCTION OF RESISTANCE BY A SIMPLE INTERCALATING DRUG DERIVED FROM THE ANTITUMOR NMHE (2N-metnyl-9-hydroxyellipticinium. Banoun H., René B., Auclair C., and Paoletti C. ; Unité de Biochimie Enzymologie, Institut Gustave Roussy, 94805 Villejuif, France.

We have synthesized a new series of cytotoxic agents derived from the antitumor NMHE (2N-methyl-9-hydroxyellipticinium) : the oxazolopyridocarbazoles (OPC) derivatives, two of them (isopropyl-OPC and isobutyl-OPC) possess an antitumoral activity on the L1210 murine leukemia in vivo (Auclair et al. (1984) J. Med. Chem. 27, 1161-1166). As NMHE, OPC derivatives interact in vitro with DNA through an intercalating process. Moreover, the fluorescence properties of OPC derivatives allows to assess they are able to intercalate in vivo bacterial nucleic acids (Banoun et al., (1985) Biochemistry 24, 701-707). Their biological properties in bacterial systems show they are simple intercalators : (1) in the S. typhimurium Ames'strains, they specifically reverse +1 frameshift mutations, (2) their toxic and mutagenic effects do not depend on the uvrB gene, (3) in E. coli, they do not induce the SOS response and (4) their mutagenic effet is not decreased in recA- mutants. Through their fluorescence properties, we have shown that their antibacterial and mutagenic effects are controlled by the in vivo accessibility to the intercalating sites in nucleic acids. Low concentrations of iPr-OPC ($\leq$4 µM) are able to induce resistance to this drug : in the presence of the drug, bacteria are able to recover exponential growth after a short time (1 to 6 generations times). They are more resistant to OPC derivatives than untreated cells. Through several growth passages in the presence of iPr-OPC, we have selected an irreversible phenotype of high resistance in S. typhimurium TA 1977 cells (R = 42). This phenotype is associated with a strong decrease (80 %) of iPr-OPC accessibility to DNA in vivo. These results suggest that simple intercalators are able to induce an adaptation process in bacteria. In tumoral cells, such a process could explain why the toxicity of OPC derivatives decrease with the time of drug contact with the cells as shown by preliminary experiments.

29 THE CAPACITY OF CULTURED ANIMAL CELLS TO INDUCE THE REACTIVATION OF DNA SYNTHESIS IN DORMANT NUCLEI IN HETEROKARYONS AND ITS DEPENDENCE ON CULTURE PROLIFERATIVE POTENTIAL. I.A.Prudovsky,Y.E.Yegorov,R.R.Gumeniuk,A.V.Zelenin
Institute of Molecular Biology,the USSR Academy of Sciences,Moscow II7334,USSR

The heterokaryons between mouse resident peritoneal macrophages and various fibroblastoid cultured cells were produced by means of PEG-induced fusion.The following cell cultures were used: I)with limited lifespan - mouse,rat and human embryo fibroblasts; 2)non-malignant,with unlimited lifespan (immortal) - NIH 3T3 and C3H IO T I/2; 3)highly malignant virus-transformed cells SV3T3.DNA synthesis in macrophage nuclei in heterokaryons was studied radioautographically at 24 - I20 hrs after fusion.Only immortal cells (non-malignant and malignant) induced the reactivation of DNA synthesis in macrophage nuclei.The cytoplasts of NIH 3T3 cells were fused with macrophages.The reactivation of DNA synthesis was detected in resultant cybrids.The Syrian hamster fibroblasts transformed with ts 239 SV40 were also used for the fusion with macrophages.At the non-permissive temperature 39,5 C° the expression of T-antigen is disturbed in these cells.In the heterokaryons with such fibroblasts the reactivation of DNA synthesis in macrophage nuclei was found at the permissive temperature 33 C°.However at 39,5 C°DNA synthesis in macrophage nuclei was not observed whereas well pronounced DNA synthesis in fibroblast nuclei was found.It is suggested that the reactivation of DNA synthesis in non-dividing differentiated cells in heterokaryons depends on the expression in culture cells of oncogenes responsible for the proliferative immortality.

30 IN VITRO REDUCTION BY BLEOMYCIN OF THYMIDINE PHOSPHORYLATION ACTIVITY IN LECTIN-STIMULATED NORMAL HUMAN LYMPHOCYTES. Abbas Sharif, Yves Goussault. INSERM U 180 - Faculté de Médecine, 45 rue des Saints-Pères - 75270 Paris cedex 06 - France.

We have investigated the effect of Bleomycin on thymidine phosphorylation in normal human cultured lymphocytes stimulated by Robinia lectin. Bleomycin is a glycopeptide possessing anti-neoplastic activity that reduces thymidine phosphorylation by decreasing the activity of thymidine kinase. Accordinly, polyacrylamide gel electrophoresis of extracts of cells incubated for 48, 72 and 96 hours showed here that this activity dropped 48, 65 and 67 % respectively. The electrophoretic profiles of thymidine kinase activity were similar but different in amplitude.

These effects of Bleomycin were confirmed firstly by direct measurement of the thymidine kinase activity, secondly by amount of ^{3}H-thymidine incorporation in the cultures before cell lysis. Both the measurement of thymidine kinase activity and ^{3}H-thymidine incorporation were correlated.

31 EXPERIMENTAL HETEROTRANSPLANTATION OF "KB" CARCINOMATOUS CELLS INTO THE EYEBALL OF RABBITS (ASSAY OF THE RELATIVE AMOUNT OF NUCLEAR DNA).
L.Schuller(1), M.Keul(2), Doina Pop D. Popa(3), L.Schuller,jr,(1),M.Kerekes(3), Maria Schuller(1),Georgeta Lazar-Keul(2),-I.Laszlo(3), F.Fodor(3), L.O.Stef(3).
(1) Laboratory of Pathological Anatomy No.II, 4300 Tirgu-Mures, P.O. Box 102, (2) Centre for Biological Research, 3400 Cluj-Napoca, Str.Republicii 48, (3) Medical Research Center, 4300 Tirgu-Mures, Str.Gh.Marinescu 40,Romania

The purpose of this study was to demonstrate the possible correlation between cholesterol-rich diet, tumor transplantation and the relative amount of nuclear DNA, in the eye of rabbits. Tumor transplantation was performed by inoculation of a suspension of carcinomatous cells, strain "KB", into the left eyeball of the rabbits (group I,II). The experiments were carried out on two groups of animals: group I received 1 g cholesterol daily, during 3 months, and group II,with tumor cells, but without cholesterol, serving as control. The relative DNA content of Feulgen-stained nuclei was measured cytophotometrically, by the two - wavelength method, and expressed in arbitrary units. The results obtained show that cholesterol-feeding produces a decrease in the nuclear DNA content in tumoral cells, as compared with the tumoral cells of animals which did not receive cholesterol. In both groups, the mean of DNA/nucleus is increased, as compared to normal cells.

32 CELL PROLIFERATION REGULATED BY CO_2
E.László, B.Novák., Department of Agricultural Chemical Technology, Technical University Budapest, H-1521 Budapest, Gellért tér 4. Hungary

Microbial and mammalian cells not proliferate when we sparged out the CO_2 from culture liquid. This means that CO_2 fixation is required for cell proliferation. CO_2 fixation need for filling up of tricarboxylic acid cycle /TCAC/, for purine and pyrimidine synthesis, and for lipid synthesis. Adding the purine and pyrimidine precursors and fatty acids into cell culture we examined the role of CO_2 in filling up of TCAC. When CO_2 concentration increased inside the cell, then increase the energy production /ATP formation/ in TCAC and respiration. This is an oscillation process, which forms a limit cycle. By oversaturated respiration chain the alternative oxydases produce hydroxyl radicals, which cause DNA damage.

We discuss the role of CO_2 in normal and abnormal cell proliferation.

33 RELEVANCE OF Cd-BANDING AND PREMATURE CENTROMERE DIVISION IN HUMAN METAPHASE FIGURES.

Luc Verschaeve, Annick Defoin, Katrien Staessen, Micheline Kirsch-Volders.
Laboratorium voor Antropogenetica, Vrije Universiteit Brussel, B-1050 Brussels, Belgium.

Kinetochore-inactivation as one of the possible mechanisms for aneuploidy was studied in human lymphocytes by two, possibly related, cytogenetic techniques: Cd-banding and premature centromere division (PCD). Cd-banding is a particular staining procedure which is presently admitted to allow to discriminate between active and inactive (or absent) kinetochores. PCD is considered to be one of the consequences of kinetochore-inactivation and the origin of at least some aneuploidy.

At first a confined study in monozygotic- and dizygotic twins did not reveal that Cd-stainability and PCD are heritable characters.

In a second approach we studied the possible role of inactivated kinetochores in aneuploidy produced after exposure of lymphocytes to aneuploidy-inducing substances. It was shown that carbaryl, methylmercury chloride, mercury chloride and arsenic trioxide did not increase the frequency of metaphase figures with Cd-negative and PCD-chromosomes. Therefor, kinetochore-inactivation does not seem to contribute to the known aneuploidy induction of these compounds.

As it is known that chromosome segregational errors are very often responsible for the occurence of early miscarriages, we also investigated some couples with recurrent abortions. Our results indicate that kinetochore-inactivation is apparently not on the origin of their abortive conceptions.

In conclusion, study in lymphocytes of Cd-banded chromosomes and PCD does not seem to be very relevant in investigations on aneugen-exposure or genetic risk populations.

34 THE EFFECT OF VERY LOW TEMPERATURES (LIQUID NITROGEN) ON MITOTIC DIVISIONS AND BIOCHEMICAL DYNAMICS IN ALLIUM CEPA HV STUTTGART DRY SEEDS.

G. Corneanu(1), R. Scorei(2), I. Enculescu(3). (1) University of Craiova, Genetics Laboratory, R-1100-Craiova, (2) Craiova Chemical Combine, R-1100-Craiova, and (3) Biofort, Sos. Caracal km.2, R-1100-Craiova, Romania.

The dry seeds of Allium cepa HV Stuttgart have been introduced (directly or frozen in water) into liquid nitrogen (-196° C) in temperature gradient of 90°C/min and kept for 3 min, 6, 24, 30 and 62 hours. The analysis of the mitotic divisions revealed a stronger action of the thermic stress both at the level of the main meristem as compared to secondary meristem, and in the case of the seeds frozen in water (the percentage of the chromosome aberrations being of 3 times higher); the chromosomes presented breakes of one-hit and two-hit type, produced both in G_1 and G_2 stages of the mitotic cycle. The strong heterochromation of some regions of the chromosomes, emphasized in prophase and metaphase, is due to the asynchronous DNA replication in the their heterochromatic regions. The dynamics of the substances from the seeds reveals their different implication in resistance to thermic stress.

35 THE EFFECT OF THE DRUG STRYCHNINE HYDROCHLORIDE ON CELL DIVISION IN MERISTEMATIC CELLS.

A.S.El-Bayoumi (1), A. Habib(2), H. Sobhi(2). (1) Botany Dept. Faculty of Science, Qatar University, Doha, Qatar, (2) Botany Dept. Faculty of Science, Ain Shams University, Cairo, Egypt.

Strychnine is a drug used as analeptic but it stimulates respiration and the cardiovascular centre only if used in convulsive doses. Repeated doses may have a cumulative effect. Roots of Allium cepa are treated with strychnine hydrochloride for 4, 8, 24, 48 and 72 h. and with different concentrations ranging from 1.97×10^{-3} M to 0.061×10^{-3}M. Cytological studies are carried out from Feulgen squash preparations. Strychnine hydrochloride induces a wide range of mitotic abnormalities. Their frequencies depend on both the duration of treatment and the concentration of the drug applied. Among these abnormalities are c-metaphase, c-anaphase, chromosome and chromatid bridges, lagging chromosomes at anaphase and telaphase. Chromosomes breaks are also induced at both metaphase and anaphase stages. Binucleate, multinucleate and micronucle are also observed. A depression in mitotic activity as indicated by the mitotic index (MI) is noticed in all treatments. The MI values decreased with the increase of concentration and duration of treatment.

36 THE EFFECT OF ENDOTOXIN /LPS/ TREATMENT ON THE MITOTIC ACT OF CHO CELLS
E. Bohdaneczky, S. U. Nagy, G. Csaba, Semmelweis University of Medicine, H-1445 Budapest, P. O. B. 370. Hungary

One hour after the LPS treatment the number of mitotic cells, the proportion of normal and atypic cell divisions and the distribution of normal, giant, two--or multinucleated cell number of the interphasis in CHO cell culture had been examined.

In the first hour following the LPS treatment the number of mitosis increased /with 80%/ and 24 hours later it was also higher /with 90%/ related to the control. The number of atypic cell divisions and the number of giant cells have also increased.

These data demonstrate the significant increasing effect of LPS on the mitotic activity of CHO cells, stimulating the appearance of atypical forms, too.

37 MITOSIS AS MAGNETOPHORESIS.
K. Uyttendaele, Lab. Dermato., Moerstraat 62, 8000 Brugge, Belgium

Consider the atomic model of Rutherford and Bohr. The electrons circulating in orbit around the nucleus of the atoms are at random distributed around the nucleus and the different magnetic fields of the different electrons neutralise each other. However if the atoms are placed in an electric field we can accept that the distribution of the electrons around the nucleus is no more at random but the electrons are now in an anode katode direction. The magnetic fields of the electrons no more neutralise each other but have an additional effect and the atoms become magnetic. By experiments in isotachophoresis we demonstrated at different international isotachophoresis societies that ions or atoms in an electric field acquire magnetic properties. The application of this low to biology can give an explanation for the mechanism of the mitosis. A chromosome at the beginning of the prophase can be considered as a conglomerate of millions of atoms in near connection with each other. In the electric field of the cell each atom and as a result the whole chromosome becomes magnetic. The magnetic chromosomes attract each other till they are in the central part of the cell. This is the mechanism of the prophase. In the metaphase all the chromosomes are in the equator of the cell with the central part--the diploid parts of the tetraploid structure--in an opposite electric field. The opposite electric field creates opposite magnetic moments in the two diploid parts of the tetraploid chromosome. These magnetic forces and the electric force excercised on the chromosome, create a couple of forces on the chromosome which result in a rotation movement of the central part of each diploid chromosome. The rotation is opposite in direction on both sides of the equator. This results in;-firstly-the disruption of the tetraploid chromosome in two identical diploid chromosomes;-secondly-the unfolding of the chromosome by rotation;-thirdly-the movement by rotation of the chromosomes to the central parts of the two half cells. This is the mechanism of metaphase and anaphase.

38 ULTRASTRUCTURAL LOCALIZATION OF Ag-NOR PROTEINS DURING INTERPHASE AND MITOSIS IN HUMAN OR MOUSE CANCEROUS CELLS.
D. Ploton (1), M. Thiry (2), M. Menager (1), A. Lepoint (2), J-J. Adnet (1) and G. Goessens (2). (1) Laboratoire d'Histologie, Faculté de Médecine, Reims, France and (2) Institut d'Histologie, Université de Liège, Belgique.

The argyrophilia of some non-histone proteins of the Nucleolar Organizer Region (AgNOR proteins) is a good cytochemical marker both of rDNA and of active substructures of the nucleolus. In this work we studied the behaviour of the Ag-NOR proteins during all the phases of mitosis in five human and murine cancerous cell-lines each characterized by a typical nucleolar morphology. The Ag-NOR proteins were stained with the one-step silver staining method followed with acetylation (D. Ploton et al. Histochemical J. 16, 897 (1984)). Our study demonstrates that nucleolar morphology is characteristic for a given cell-line considering variations in the relative amount and disposition of the nucleolar components. Ag-NOR proteins are strictly localized within fibrillar centres (F.C.) and dense fibrillar component (D.F.C.). The behaviour of the Ag-NOR proteins during mitosis is very similar in the five cell-lines. During prophase the Ag-NOR proteins come in close contact with some chromosomes and a typical polarity is shown : chromosome, F.C. and D.F.C. Then F.C. and D.F.C. aggregate to constitute silver-stained roundish structures which then associate in pairs or in triplets to constitute the metaphasic NORs integrated in part within dips at the periphery of the chromosomes. During anaphase and telophase large and small spherical argyrophilic structures are seen : they correspond respectively to the metaphasic NORs and to numerous small structures which appear "de novo" within RNP material localized between the chromosomes and then fused to form the prenucleolar bodies during late telophase. This work suggests that when rRNA synthesis is impaired during mitosis the inactive NORs get a structure and a localization which are typical of the phase of mitosis but not of the cell-line. At the opposite, F.C. and D.F.C. are probably two aspects of the active NORs which typical spatial distribution gives to the interphasic nucleolus its characteristic morphology.

39 X-RAY MICROANALYTIC MEASUREMENTS OF THE $Na^+:K^+$ RATIO IN HUMAN LARYNGEAL TUMORS

Z. Szállási (1), I. Zs.-Nagy (1), L. Tóth (2) and I. Lampé (2). (1) F.Verzár International Laboratory for Experimental Gerontology (VILEG), Hungarian Section and (2) Department of Oto-Rhino-Laryngology, University Medical School, H-4012 Debrecen, Hungary

Energy-dispersive X-ray microanalysis was performed on human biopsy materials taken during laryngoscopic interventions from 18 cases. The biopsies were carried out during the first diagnostic interventions before any cytostatic treatment or radiation therapy has been applied. The removed tissue pieces were divided in two parts. One of them was used for pathohistological analysis, the other one was processed for X-ray microanalysis by the freeze-fracture freeze-drying method. Four of the cases to be of benign character, whereas the rest contained carcinoma planocellulare keratoides or non-keratoides as revealed by the pathohistologic analysis. Bulk specimen Energy dispersive X-ray microanalysis of 135 cells from the benign tissue samples revealed an average $Na^+:K^+$ molar ratio of 0.13 $\pm$ 0.01 (S.E.M.) in the intracellular space, with a regular Gaussian distribution. In the cases of carcinoma 641 cells were measured, the average of the same ratio was 0.67 $\pm$ 0.03 (S.E.M.) due mostly to an increase of the Na^+ content. The distribution of data was apparently not normal in the cancerous samples. The histograms were compared by the χ^2 test. This type of analysis revealed that the average intracellular $Na^+:K^+$ molar ratio increased about 5-fold in the cancerous samples. The results are interpreted in terms of the membrane hypothesis of mitogenesis (Cone, J. theor. Biol. 30, 151-181, 1971) according to which the Na^+-influx is a necessary prerequisite for the initiation of mitosis (Koch and Leffert,Cell, 18, 153-163, 1979). Some further comments will be made on the interpretation of the Amiloride action on this process in the light of some recent results.

40 IMMUNOCYTOCHEMICAL INVESTIGATION OF ANTIBODIES AGAINST ANTIGENS OF SYNAPTONEMAL COMPLEXES

O.L. Kolomiets, S.Ya. Dadashev, G.G. Gorach. N.I. Vavilov Institute of General Genetics, USSR Academy of Sciences, B-333 Moscow 117809, USSR

Antibodies against antigens of synaptonemal complexes (SC) were tested by the method of indirect immunofluorescence. Spread SCs from mouse spermatocytes served as a substratum for immunocytochemical studies. Sera obtained from control (preimmunized) mice and from mice immunized by isolated SCs were used as primary sera. Polyclonal monospecific affinity-purified rabbit immunoglobulins against mouse immunoglobulins (IgG) were used as a secondary serum. Mouse SCs were stained by antibodies from both immunized and control animals. The brightest fluorescence was observed in terminal dots. Autosomal SCs and XY-SCs were stained more intensively than the asynaptic ends of X and Y chromosomes. Horse and rat SCs were stained by sera from the immunized and control mice. Data on the existence of a correlation between the titers of autoantibodies against SC antigens in the control animals and the stage of their ontogenesis have been obtained. We suppose that the antibodies against SC antigens that are present in the serum of the control animals play the role of autoantibodies - witnesses of natural degeneration of spermatocytes. Maximum degeneration is known to occur in mammals just at early stages of meiosis, and part of these cells is phagocytized by Sertoli cells. The similarity of the antigenic structure in animals belonging to different taxonomic groups is probably determined by the phylogenetic age of the SC and by the identity of its ultrastructure in the animals studied.

41 BIOCHEMICAL STRUCTURE OF ISOLATED SYNAPTONEMAL COMPLEXES FROM MOUSE SPERMATOCYTES

G.G. Gorach, S.Ya. Dadashev, Yu.F. Bogdanov, O.L. Kolomiets. N.I. Vavilov Institute of General Genetics, USSR Academy of Sciences, B-333 Moscow 117809, USSR

Isolated mouse synaptonemal complexes (SC) were obtained by hydrolyzing pachytene nuclei with DNase I and II and subsequent purification from subnuclear admixtures in stepwise sucrose concentration gradient. The isolated SCs contain 90% of protein, 5% of hexoses, 3% of DNA and 2% of RNA. A quarter of proteins is represented by histones. The rest of proteins are polypeptides from 12.5 kD to 90 kD. Glycoproteid with a molecular mass of 12 kD is a component of SC lateral elements. It is distributed over the whole length of the SC and seems to play an important role in the preservation and organization of the SC structure. DNA related to the SC structure may be divided into two types of fragments: large fragments (over 2000 base pairs) and short fragments which are components of DNP and which cannot be hydrolyzed without preliminary removal of proteins and sugars by proteinase K and hyaluronidase. RNA is present in the SCs as RNP particles with a molecular mass of 31 kD. These particles are accumulated in the nuclei during pachytene and are absent in the nuclei of spermatocytes at other stages of meiosis and in spermatides. The conditions of SC dissociation were studied and it has been established that ionic and hydrophilic-hydrophobic bonds as well as S=S bridges play an essential role in the integrity of the SC structure.

42 ULTRASTRUCTURAL ASPECTS OF NUCLEOLOIDS IN THE OLEA EUROPAEA (L) MICROSPORE.

Rodríguez García, M.I. & M.C. Fernández Fernández. Estación Experimental del Zaidín, CSIC, Granada, Spain.

Bodies with nucleolus like characteristics have been illustrated and described in the meiocyte cytoplasm of Lilium with the term "nucleoloids" (1). There is evidence that the presence of cytoplasmic nucleoloids is commonplace in plant microsporogenesis (2,3,4). However, with the exeption of Lilium (1, 5) no other studies have appeared on the fine structure of nucleoloids and their relationship with changes in the meiocyte ribosome population of other angiosperms.

This study describes the presence of nucleolus-like bodies in the microspore cytoplasm of Olea europaea and its ultrastructure along with the results obtained by means of the selective technique for RNA (6), used to confirm the ribonucleoproteic nature of these bodies. A direct relationship was also established between the presence and behaviour of the nucleoloids and the ribosome population of the microspore. In the newly-formed tetrad, when the nucleoloids first appear, ribosomes are extremely scarce in the cytoplasm; as the ribosomes increase in number in the early microspore, the granular component of the necleoloids appears to undergo desaggragation. At this time the nucleoloids are surrounded by a great number of polyribosomes. Finally, in the vacuolated microspore, the nucleoloids disappear simultaneously with a notable rise in ribosome population. These observations are in agreement with the role previously attributed to nucleoloids in the replenishment of the ribosome population following meiosis (1,5).

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43 RELATIONSHIP BETWEEN DESIALILATION OF PLASMA MEMBRANE AND DNA SYNTHESIS DURING PREREPLICATIVE PHASE OF HEPATOCELLULAR PROLIFERATION.

Mª J. Coll, M. Soriano, Mª J. Pujol, O. Bachs and C. Enrich. Dept. Histology and Cell Biology. Fac. Medicine. University Barcelona. Spain.

It has been measured the content of sialic acid bound to the sinusoidal region of plasma membrane during prereplicative phase after partial hepatectomy and also after the intravenous injection of a solution containing triiodothyronine, amino acids, glucagon and heparin (T.A.G.H. solution). The results obtained show that an important decrease in sialic acid content is produced in both systems.

In order to know if sialidase activity is involved on the decrease of sialic acid content during liver regeneration, it has been studied the activity of sinusoidal plasma membrane sialidases during the prereplicative phase after partial hepatectomy. No modifications of sialidase activity were detected during this period of time indicating that this decrease in sialic acid content has to be produced by others mechanisms as for instance diminution in the synthesis of precursor molecules.

On the other hand due to the importance of Ca^{2+}- calmodulin complex in the activation of the hepatic cells proliferation it has been studied the possible implication of this complex on the initiation of DNA synthesis and also on the loss of sialic acid observing the effect of Trifluoperazine (inhibitor of Ca^{2+} - calmodulin complex) during the prereplicative phase of liver regeneration. Results we have obtained show that Trifluoperazine delays aproximately 12 hours both events respect to the normal responses observed in not treated regenerating rats.

These results suggest that DNA synthesis and the decrease in the amount of sialic acid bound to sinusoidal region of plasma membrane are dependent on previous surge of calmodulin observed at 8-12 hours after partial hepatectomy.

44 THE MECHANISM OF POSTPROPHASE NUCLEOLUS FORMATION

Bogdan Wróbel, Alicja Górska-Brylass. Department of Plant Cytology and Genetics Institute of Biology, Copernicus University, Gagarina 9 87-100 Toruń Poland.

The experiments were carried in synchronized /0,2% caffeine/ population of binucleate meristematic cells of Lupinus luteus L. roots, species in which postprophase /persistent/ nucleolus was found in 50% of metaphase cells. During the period G_2 of cell life cycle the metabolism of nucleic acid was interrupted and it was observed that: 1/ Blocking the availability of the template in the presence of actinomycin D or ethidium bromide does not influence the delay of nucleolus disappearance. 2/ Disturbance of maturation of ribosomal RNA inducted with 5-fluorodesoxyuridin or 5-fluorouracil results in increase in number of cells containing postprophase nucleolus. The effects of these inhibitors are eliminated by the plant hormone - gibberellin /GA_3/.

45 SIMULTANEOUS STATIC CYTOFLUORIMETRIC ANALYSIS OF DNA AND PROTEIN CONTENT OF NUCLEI RELEASED FROM PEA GERMINATING EMBRYOS.

Sergio Sgorbati, Marisa Levi, Maria Lusardi, Elio Sparvoli. University of Milan, Department of Biology and Unit for Cell and Molecular Biology of Plants (CNR), (I-20133 Milano, Italy).

In the past years many studies were carried out by biologists on animal cell cultures, with the use of biparametric flow cytometric techniques, in order to have major insight in studying G1 and G2 cell cycle compartments. In spite of the interest of such studies also for the analysis of the plant cell cycle, only few cytophotometric works were carried out on squashes or sections of plant tissues. We measured DNA and protein content of nuclei released from root apices, during germination of pea embryos, before the resumption of DNA synthesis. We employed DAPI and FITC as specific fluorochromes for DNA and proteins respectively. We analysed samples of about 600 nuclei; the protein content of 4C nuclei was nearly twice that of 2C nuclei, in spite of a great variability of protein content within the two DNA classes. The first results appear also promising in individuating different compartments in the G1 and G2 phases, as obtained in animal cells. Advantages of this method with respect to cytophotometry are: simple and less expensive instrumental configuration; analysis made directly on smears of nuclei randomly released from a number of different embryos; no necessity of cytoplasmatic mask, as in squash preparations, or nuclei selection, as in sections. With this method we are now studying variations of nuclear protein content in embryo root during early germination, in order to get further informations about critical conditions that allow the onset of DNA synthesis or mitosis during the first cell cycle in this natural system.

46 REGULATORY SEQUENCES IN STEROID CONTROLLED GENES.

Günther Schütz, Richard Miksicek, Michael Jantzen, Peter Becker, Gerd Klock, Bernd Gloss, Uwe Strähle and Wolfgang Schmid. Institute of Cell and Tumor Biology, German Cancer Research Center, Im Neuenheimer Feld 280, D - 6900 Heidelberg, F.R.G.

Analysis of expression of lysozyme gene recombinants in oviduct cells has allowed us to identify sequences required for progesterone and glucocorticoid induction. These sequences represent binding sites for both the glucocorticoid and progesterone receptors. Single and clustered point mutations have been generated to determine the essential features recognized by these two distinct steroid receptors. Lysozyme gene sequences containing the receptor binding sites when fused to the thymidine kinase promoter fail to show regulation in non-oviduct cells, but evidence a strong constitutive enhancer-like activity in mammary gland cells.

The expression of the tyrosineaminotransferase (TAT) gene in liver parenchymal cells is controlled by glucocorticoids and cAMP. Analysis of expression of fusion genes containing 5'-flanking sequences of the TAT gene after transfer into rat hepatoma cells and fibroblasts allowed identification of glucocorticoid control elements located 2500 bp upstream of the initiation site of transcription. The sequences recognized by the glucocorticoid receptor have been defined by DNaseI footprinting and methylation protection experiments in vitro. Methylating the DNA in intact hepatoma cells with dimethylsulfate and visualizing the methylation pattern with the genomic sequencing technique, we observe distinct changes in the receptor binding sites after induction. cAMP induces transcription of the TAT gene by an independent mechanism involving the cAMP dependent protein kinase.

47 HEPATOCYTE SPECIFIC EXPRESSION OF HUMAN CLONED GENES

Riccardo Cortese, European Molecular Biology Laboratory, Meyerhofstrasse 1, 6900 Heidelberg, F.R.G.

A large number of proteins are specifically synthesized in the hepatocyte. Only the adult liver expresses the complete repertoire of functions which are required at various stages during development. There is therefore a complex series of regulatory mechanisms responsible for the maintenance of the differentiated state and for the developmental and physiological variations in the pattern of gene expression. Human hepatoma cell lines HepG2 and Hep3B display a pattern of gene expression similar to adult and fetal liver, respectively; in contrast cultured fibroblasts or HeLa cells do not express most of the liver specific genes. We have used these cell lines for transfection experiments with cloned human liver specific genes. DNA segments coding for alpha1-antitrypsin and retinol binding protein (two proteins synthesized both in fetal and adult liver) are expressed in the hepatoma cell lines HepG2 and Hep3B, but not in HeLa cells or fibroblasts. A DNA segment coding for haptoglobin (a protein synthesized only after birth) is only expressed in the hepatoma cell line HepG2 but not in Hep3B nor in non hepatic cell lines. The information for tissue specific expression is located in the 5′ flanking region of all three genes. In vivo competition experiments show that these DNA segments bind to a common, apparently limiting, transacting factor. Conventional techniques (Bal deletions, site directed mutagenesis, etc.) have been used to precisely identify the DNA sequences responsible for these effects. The emerging picture, is, at the moment, complex: we have identified multiple, separate transcriptional signals, essential for maximal promoter activation and tissue specific expression. Some of these signals show a negative effect on transcription in fibroblast cell lines.

48 HnRNPs CARRYING HnRNA AND snRNA: STRUCTURES INVOLVED IN mRNA MATURATION.

Apostolia Guialis, Amalia Dangli, Maria Hatzoglou, Eftichia Adamtziki, Anastasia Arvanitopoulou and Constantine E. Sekeris. Institute of Biological Research, National Hellenic Research Foundation, 48 Vassileos Constantinou, Athens, Greece.

Maturation of HnRNA (pre-mRNA) is a complicated, multistep process of the eucaryotic cell nucleus. That HnRNA does not appear free in the nucleus, but in association with a specific set of proteins in the form of nuclear ribonucleoprotein particles (HnRNPs), is also well documented. An important role in pre-mRNA splicing has been ascribed to snRNA molecules that exist either as discrete ribonucleoprotein particles with approximate S values of 10S or in association with HnRNP complexes. We have been interested in both the structural and functional aspects of HnRNPs and to this extent we have isolated and characterized different HnRNP populations as components of the intact, pre-existing, in vivo structures. By a combination of biochemical and immunochemical technics we have characterized these HnRNP structures with respect to the protein, snRNA and specific RNA sequences they contain. In particular, we are interested in a discrete RNP sub-population that cosediments on sucrose gradients with HnRNP monomers of approximately 40S and contains snRNA and proteins other than the core proteins of monoparticles. This RNP structure (MI) has been separated from the HnRNP -monoparticles (MII) by electrophoresis in agarose (Guialis, et al, FEBS Letters, 151, 127 (1983)). In this respect this novel structure does not appear to be an integral part of the monoparticle, but very probably associates with HnRNPs by virtue of base pairing between snRNA of MI and HnRNA of the HnRNPs (Sekeris and Guialis, in The Cell Nucleus,

Bush H. ed. Vol. 8, 247, 1981). We regard this structure as the "activated" form of snRNPs, carrying the respective enzymes and factors for the maturation process. Antibodies to the proteins of MI are now in the process of being raised. In addition, we have isolated a 70-110S RNP, composed of HnRNA, snRNA and a specific set of proteins (Hatzoglou, et al. Expt. Cell Res., 157, 227 (1985) differing from those of MI or MII. This structure, in contrast to HnRNP-polymers, is resistant to RNAse action, but its S value decreases in high salt. We are also examining the mode of association of snRNA to HnRNP particles. Our results indicate differences in the way specific snRNP:HnRNP assemblies are formed. For example, U_1-snRNP is mainly bound in a loose, easily extractable form. Only a small portion is tightly bound to HnRNP, which can be regarded as the form actively engaged in splicing. On the other hand U_5-snRNP is found highly enriched on monoparticles in a tightly bound form. Finally, we are establishing an in vitro reconstituted system encompassing these different RNP components, carrying out some or all of the steps involved in specific pre-mRNA-processing.

49 CONTROL OF RIBOSOMAL PROTEIN GENE EXPRESSION IN XENOPUS.

Francesco Amaldi(1), Elena Beccari(2), Irene Bozzoni(2), Paolo Mariottini(1), Paola Pierandrei-Amaldi(3). (1) Dipartimento di Biologia, II Università di Roma "Tor Vergata", via Orazio Raimondo, 00173 Roma; (2) Centro Acidi Nucleici C.N.R., p.le A.Moro 5, 00185 Roma; (3) Istituto di Biologia Cellulare C.N.R., via Romagnosi 18/A, 00196 Roma, Italy.

The genes coding for ribosomal proteins (r-proteins) are present in two copies per haploid genome in Xenopus laevis. Some of these genes (for r-proteins L1, L14 and S19) have been isolated and their structure analyzed. They contain several introns and are characterized by a peculiar 5' end; in fact the transcription initiation sites of these genes are located within stretches of 15-20 pyrimidines and are preceded by a non canonical TATA-box.

We have also studied the expression of r-protein genes during oogenesis and during development of Xenopus normal and anucleolate embryos (these O-nu mutants have a deletion of the r-RNA gene cluster). We have analyzed: 1) the synthesis and accumulation of rp-mRNA (mRNA specific for r-proteins), 2) the distribution of rp-mRNA on polysomes and mRNP, 3) the translational activity of these mRNAs and 4) the stability of the newly synthesized r-proteins. Another approach to the study of r-protein gene regulation has been the microinjection of cloned genes for r-proteins L1 and L14 into the GV of Xenopus oocytes or in fertilized eggs, followed by the analysis of the effects of these gene dosage changes at the transcriptional, post-transcriptional and translational levels. The effect of microinjection of an excess of purified r-proteins on the translation of their own mRNAs has also been studied. The overall conclusion of these studies is that at least two types of regulation of r-protein synthesis are operating in the Xenopus system. The first works at the translational level by changing the polysome/mRNP distribution of rp-mRNA and seems to respond to the need of new ribosomes. The second works at the post-transcriptional level controlling the stability of the transcript and might be autogenously affected by an overproduction of r-proteins with respect to the amount needed for ribosome assembly. We have shown that the post-transcriptional regulation observed in the L1 gene dosage experiments involves a block of a specific step of L1 transcript splicing, resulting in the accumulation of a partially processed RNA precursor still containing two of the nine introns sequences of the L1 primary transcript.

The sequence analysis of the L1 gene has also revealed that four of the nine introns have a remarkable sequence homology. The region of homology spans 60 nucleotides and comprises several boxes with 100% homology, the longest, box 3, being of 16 nucleotides. Computer analysis has revealed that perfect sequence complementarity exists between 13 nucleotides of box 3 and the 28S ribosomal RNA in a region which is conserved in all Eukaryotes. The high evolutionary conservation of these sequences suggests a possilble base pairing interaction between intron sequences of r-protein L1 transcripts and 28S rRNA, which might be important for r-protein - rRNA synthesis coordination.

50 T-CELL RECEPTOR GENE STRUCTURE AND EXPRESSION.
Zlatko Dembic, Werner Haas, Siegfried Weiss, James McCubrey, Hansruedi Kiefer, Harald von Boehmer and Michael Steinmetz, Basel Institute for Immunology, CH-4058 Basel, Switzerland

Within the immune system of vertebrates, T-lymphocytes have a variety of important roles, like the direct killing of virally infected cells (by cytotoxic T cells) and either amplification (by helper T cells) or inhibition (by suppressor T cells) of the response to antigen. Recognition of foreign entities by T cells can occur only in the context of autologous cell-surface protein encoded by major histocompatibility complex (MHC). This 'dual recognition' by T cells raises the question whether T-cell antigen receptors have two binding sites, one for foreign antigen and one for the self MHC molecule.

The T-cell receptor is a multimeric complex. The putative recognition structure is a polymorphic disulfate-linked heterodimer composed of α and β chains. The heterodimer is associated on the cell surface with a group of nonpolymorphic proteins (the subunits of the T3 molecule), which might be important for effector functions.

The genes for α and β chains are divided, like immunoglobulin genes, in variable (V), joining (J) and constant (C) regions which rearrange during T-cell ontogeny. A third gene family called γ, closely related to α and β, has been found to be rearranged and transcribed in T cells but its function is unknown.

The structural analysis of the T cell receptor genes has failed to reveal a major difference between the T-cell receptor polypeptides and the related immunoglobulin molecules which could account for the 'dual specificity' of T cells. The most straightforward way to investigate this question is to transfer T-cell receptor genes between T cells of different specificities and to analyse the transfectants for the gain of new specificities.

To find out whether α and β chains are sufficient for antigen and MHC recognition, we isolated functional T-cell receptor genes from one mouse cytotoxic T-cell clone and transferred them into another T-cell with different specificity. The α and β alleles were isolated from the T-cell clone BDFL1.1, which lyses targets carrying the D^d MHC molecule and coupled with fluorescein (FL) as antigen. The BDFL α and β genes were ligated with a selectable marker (neomycin resistance gene) and transferred by protoplast fusion into the T-cell hybridoma SPH1.3 (a killer T cell specific for the hapten 3-(p-sulfophenyldiazo)-4-hydroxyphenyl acetic acid (SP) and the K^k MHC molecule). One transfectant was found to express the introduced α and β chain genes at the RNA level. When tested with the appropriate target cells, it showed the host cell specificity ($SP+K^k$) and, in addition, the specificity of the donor cell ($FL+D^d$). We conclude, therefore, that in our system the T-cell receptor α and β chains are sufficient to transfer MHC-restricted antigen specificity.

51 SEQUENCES AND FACTORS INVOLVED IN SPECIFIC INITIATION AND TERMINATION OF MOUSE rDNA TRANSCRIPTION.
Ingrid Grummt, Joachim Clos, Detlev Buttgereit and Ursula Maier. Institut für Biochemie der Universität Würzburg, Röntgenring 11, D-8700 Würzburg, F.R.G.

We have purified two factors that are required for efficient and accurate transcription initiation by RNA polymerase I. Growth-dependent regulation of rRNA transcription is mediated by factor TIF-IA, the amount or activity of which fluctuates according to the physiological state of the cells. The accuracy of the initiation reaction is brought about by a species-specific DNA-binding protein (TIF-IB) that interacts with defined rDNA sequences upstream of the initiation site (from -6 to -21). Single nucleotides essential for binding of TIF-IB have been identified by site-specific mutagenesis. Furthermore we show that in vivo RNA polymerase I terminates transcription 565 bp downstream of the 3'end of mature 28S RNA. This specific termination also occurs in vitro. RNA molecules with authentic 3'ends are transcribed from ribosomal minigene constructs provided the templates retain a minimal length of downstream spacer sequences. Analysis of the nucleotide sequence of the region of transcription termination reveals a set of repetitive structural elements which consist of 15 bp conserved nucleotides surrounded by stretches of pyrimidines. Termination in vivo occurs within the first element. This termination site is preferentially used in vivo at low template concentrations. At increasing DNA concentrations another termination site within the second repetitive element is used. Competition experiments with defined 3'terminal fragments suggest that transcription termination by RNA polymerase I requires the interaction of some factor(s) with the repetitive structural elements in the 3' nontranscribed spacer.

52 NUCLEASE HYPERSENSITIVE STRUCTURES IN THE HSP 70 HEAT SHOCK CHROMATIN OF DROSOPHILA MELANOGASTER

Andor Udvardy /1/, Paul Schedl /2/. /1/ Biological Research Center of the Hungarian Academy of Sciences. H-6701 Szeged, POB 521, Hungary and /2/ Department of Biology, Princeton University. 08544 Princeton USA.

The chromatin organization of a 30 kb DNA sequence surrounding the hsp 70 heat shock genes at the 87A7 cytogenetic region of Drosophila melanogaster was examined. Our results indicate that this locus has a complex chromatin organization. The hsp 70 genes are characterized by multiphasic nucleosomal arrangement. Complex nuclease hypersensitive structures were detected at the 5′ end of the hsp 70 genes. In the intergene spacer separating the two divergently oriented hsp 70 genes nucleosomes are aligned with respect to the underlying DNA. The 3′ flanking spacer at both the proximal and the distal side shows multiphasic nucleosomal arrangement. Heat induction causes highly specific alterations in the chromatin throughout the locus. There are major changes in the 5′ nuclease hypersensitive structures, within the heat shock transcription units and in both the upstream and downstream flanking spcer.

Specific chromatin structures /SCS/, characterized by a complex set of nuclease hypersensitive sites form the proximal and distal boundaries of the heat shock locus. Highly specific rearrangements of the nuclease hypersensitive sites, observed after heat induction indicate the functional involvement of these SCS formations in the heat shock locus.

The 5′ end of the heat shock genes and the SCS structures at the boundaries of the heat shock locus are preferentially cleaved not only by DNase I and micrococcal nuclease, but also by the single strand specific S1 nuclease. The enhanced S1 nuclease sensitivity of DNA sequences at the 5′ end of the heat shock gene is retained in supercoiled, but not in linear plasmid, suggesting that torsional strain is responsible for the generation of S1 nuclease hypersensitive sites within the chromatin. After heat induction, there is a marked reduction in the yield of the prominent 5′ S1 nuclease fragments, while the entire hsp 70 gene, as well as the spacer DNA just downstream from the 3′ end of the gene, which are highly resistant in the uninduced chromatin, becomes highly sensitive for S1 nuclease. Gross rearrangement was observed in the S1 nuclease cleavage pattern of the SCS structures after heat induction.

To study the role of torsional strain in maintaining the S1 nuclease hypersensitivity of the promoter and SCS structures, the effect of novobiocin, an inhibitor of DNA topoisomerase II was examined on the heat inducibility of the hsp 70 genes, and the chromatin structural changes described above. If novobiocin is added before heat induction, there is no detectable expression of the heat shock genes. Moreover, the antibiotic prevents the alterations of S1 nuclease cleavage pattern of the chromatin which normally accompany heat induction. When novobiocin is added after induction, transcription appears to be rapidly turned off, and the S1 nuclease cleavage pattern of the chromatin in the heat shock locus is "fixed" in an "active" configuration. Novobiocin also prevents the re-establishment of the preinduced S1 nuclease pattern which occurs during recovery from heat shock. These findings indicate the significance of torsional changes of DNA in the regulation of gene expression, and in the establishment of active chromatin configuration. This assumption is further supported by our observation that the main localization of topoisomerase II within the heat shock chromatin coincides with the major S1 hypersensitive sites.

53 TISSUE-SPECIFIC EXPRESSION OF THE RAT ALBUMIN GENE: GENETIC CONTROL OF ITS EXTINCTION IN MICROCELL HYBRIDS. Christine Petit, Jacqueline Levilliers, Marie-Odile Ott and Mary C. WEISS. Centre de Génétique Moléculaire, Centre National de la Recherche Scientifique, 91190 Gif-Sur-Yvette ; Present address: Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15.

Analysis of the expression of differentiated functions in hybrid cells formed by the fusion of cells of different histotypes has indicated that somatic cells produce trans-acting regulators (extinguishers) that prevent the expression of functions foreign to their own differentiation. In microcell hybrids between albumin-producing rat hepatoma cells and microcells of mouse fibroblast L cells, the extinction of albumin production is directly related to the presence of a single specific chromosome of the mouse fibroblast parent. The expression of several other hepatic functions is not affected when this chromosome is present. Transfection of these hybrids with a recombinant plasmid containing a tissue-specific control element of the upstream region of the rat albumin gene linked to coding sequences of the chloramphenicol acetyltransferase gene reveals that extinction acts on or via this cis-control element.

54 ACTIVATION OF ALPHA-FETOPROTEIN SYNTHESIS IN RAT HEPATOMA CELLS WITH REDUCED SENSITIVITY TO DEXAMETHASONE. Aniko Venetianer.
Institute of Genetics, Biological Research Center, Hungarian Academy of Sciences, Szeged POB 521, Hungary.

The Faza 967 differentiated, dexamethasone-sensitive cell line of Reuber rat hepatoma cells does not synthesize detectable amounts of alpha-fetoprotein (AFP), but produces albumin. AFP production has been activated in differentiated, reduced glucocorticoid-sensitivity variants of Faza 967 cells upon culture for several months in the presence of high concentration of dexamethasone. The stability of AFP production differed among the variants, while albumin synthesis did not change indicating that the regulation of these two genes is not coordinated. We have shown by molecular hybridization that AFP message could not be detected in the AFP non-producing cells suggesting that the lack of AFP synthesis most probably originates from a transcriptional block of the AFP gene or from an unstability of the AFP message. A good correlation could be demonstrated between the AFP gene activation and hypomethylation of two MspI sites in and around the S' end of the gene. We have also found correlation between the expression of albumin gene and hypomethylation of one MspI site in the 5' end of this gene. DNAseI hypersensitive sites around the 5' end of AFP and albumin genes were also characterized and differences were detected in chromatin structure of AFP and albumin producing and non-producing cells. AFP-producing and non-producing variants of Faza 967 cells offer a valuable model system for studying the regulatory mechanisms involved in activation and inactivation of the gene coding for the oncodevelopmental protein AFP.

55 MUTANT GLUCOCORTICOID RECEPTORS

Ulrich Gehring, Institut für Biologische Chemie, Universität Heidelberg, D-6900 Heidelberg, Im Neuenheimer Feld 501, FRG

In various cells of lymphatic origin glucocorticoid hormones elicit drastic cell inhibitory responses which culminate in cell lysis. This effect can be used for selecting resistant variants from populations of responsive wild-type cells. Three types of glucocorticoid receptor abnormities have been found amongst resistant cell clones of mouse lymphoma cells. These mutant receptors have either defects in their respective hormone binding and DNA interaction sites or they have lost a polypeptide domain which functions in modulating the receptor's interaction with DNA. Biochemical and immunochemical methods have been used for characterizing these receptor mutants and for comparing them with the wild-type molecule.

56 REGULATION OF MAJOR HISTOCOMPATIBILITY GENE BY INTERFERON
A MODEL TO ANALYZE EUKARIOTIC GENE REGULATION

M.FELLOUS and F. ROSA
Immunogénétique Humaine - Institut Pasteur
25 rue du Docteur Roux - 75724 PARIS CEDEX 15 - FRANCE

Interferon (IFN) enhance cell surface level of HLA class I and class II HLA molecule as well as beta2 microglobulin. This increment is associated with an enhancement of the corresponding messanger RNA. Using isolated nuclei and run off experiment, we were able to quantify directly the transcription rate of HLA class I, β2 microglobulin and HLA class II genes we demonstrate that IFN-β and γ enhances the transcription rate of HLA class I and β2 microglobulin gene in a 3-4 fold ratio after one hour of treatment. The transcription of HLA class II gene behave differently. IFN γ appears to act as a specific regulator of HLA class II genes; it regulate these gene at a posttranscriptional level in vitro. Transfection of human or mouse class I histocompatibility gene strongly suggest the importance of the 5' promotor region in the IFN regulation.Genetic control of IFN-β and γ receptor have been analyzed using man/mouse hybrid. They show that receptor pf IFN-β and γ are controled by different human chromosome : chromosome 21 and 18 respectively. Finally the biological significance of HLA interferon regulation will be discussed.

57 DEVELOPMENT OF THE DNA-METHYLASE SYSTEM AS A FUNCTION OF THE HELA CELL CYCLE

Delfini C. (1), Lo Presti E. (1), Eremenko T. (2) and Volpe P. (2). (1) Istituto Superiore di Sanità, Rome, and (2) Institute of Experimental Medicine, CNR, Rome, Italy

The major part of DNA-methylation occurs during the S-phase, semiconservatively involving the newly synthesized chains (1). Such methylation was thought to be performed by a maintenance enzyme (2). However, the existence of at least two DNA-methylases was suggested functioning in early S on GC-rich and in late S on AT-rich sequences, correspondingly (3). Moreover, some extra-S-phase DNA-methylation was found (1). An investigation was thus undertaken to ascertain whether the DNA-methylase activity is due always to the same enzyme in synchronized cells or may belong to different DNA-methylases appearing at given cell cycle stages. The data reported here suggest that two peaks of DNA-methylase activity (the first small, the second large) appear in S. Another large peak of DNA-methylase activity appears in late G_1. In crude samples, the main S- and G_1-methylation reactions follow different patterns against pH. This supports the hypothesis that the correspondent methylases, if they are more than one (3), might accompany during interphase the DNA-polymerases alfa or beta(4); accumulation of a specific DNA-methylase in late G_1 might account for complection of the DNA "repair-modification" enzyme system (5), before replication.

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58 STUDIES ON THE REGULATION OF THE TISSUE- AND DEVELOPMENTAL STAGE-SPECIFIC EXPRESSION OF THE RAT EMBRYONIC MYOSIN HEAVY CHAIN GENE. Emanuel E. Strehler, Vijak Mahdavi, Marie-Antoinette Strehler-Page and Bernardo Nadal-Ginard. Dept. Mol. & Cell. Cardiology, Children's Hospital Med. Ctr., Harvard Medical School, 300 Longwood Ave., Boston, MA 02115, U.S.A.

Vertebrate sarcomeric myosin heavy chain (MHC) isoforms are encoded by a multigene family whose members are expressed in a tissue-specific and developmentally regulated manner. In the rat, each MHC gene comprises approx. 25 kb of DNA and shows a highly complex exon-intron structure (e.g., 41 exons in the embryonic gene). In order to identify the sequences involved in their regulation of expression MHC minigenes were constructed and introduced into myogenic and nonmyogenic cells by the calcium phosphate transfection procedure. Transient MHC gene expression was analyzed by S1-nuclease mapping and Northern blotting of total cellular RNA 48 hours after transfection. A rat embryonic MHC minigene consisting of 2.6 kb of 5' flanking sequence, exons 1 to 3 and 37 to 42 (thus carrying a 20 kb intragenic deletion) was still accurately expressed in differentiating mouse skeletal muscle C2 cells, but not in undifferentiated C2 myoblasts or nonmuscle cells. In contrast, a similar construct made from the α-cardiac MHC gene was not expressed in any of the above cell types. However, a hybrid minigene consisting of the embryonic MHC 5' portion and the α- or β-cardiac 3' end was expressed in an embryonic skeletal muscle-specific manner demonstrating the importance of the 5' sequences for correct MHC gene expression. Deletion of sequences upstream of position -300 from the cap site reduced the expression of the embryonic MHC minigene to a very low level. Interestingly, insertion of an SV40 enhancer into the first intron or downstream from the 3' end of the MHC minigenes rendered these constructs - even the α-cardiac minigene - expressible at a very high level, but again only in differentiated muscle cells. From these results we conclude that more than one type of tissue-specific regulatory sequences may be present in the 5' flanking region of the rat embryonic MHC gene. We are currently studying these sequences in more detail to define the possible tissue-specific enhancer and/or promoter elements of MHC genes.

59 STUDIES ON INTER-KINGDOM HETEROKARYONS. E.C. Cocking, M.R. Davey, A.R. Underwood, R.H. Clothier[1] and M. Balls[1]. Department of Botany and [1]Department of Human Morphology, University of Nottingham, NG7 2RD, U.K.

Plant protoplasts and animal cells have been fused to produce viable heterokaryons. Immunofluorescence showed retention of both animal and plant specific membrane components in Xenopus cell-carrot protoplasts for at least two weeks following fusion. Cultured heterokaryons exhibit both plant and animal cell functions, e.g. Petunia protoplasts electrofused with Friend cells regenerate cell walls and show DMSO stimulated haemoglobin synthesis when cultured in plant protoplast media (Salhani, N. et al., Protoplasma 126 30, 1985). Gene transfer and expression has been confirmed following culture of heterokaryons under conditions which select preferentially for growth of heterokaryons as either animal or plant cells. Mammalian cells resistant to the antibiotic G418 resulted from fusion of plasmid-transformed yeast cells with Chinese Hamster cells when heterokaryons were cultured in animal cell medium. Similarly, G418 resistant yeast cells were produced when heterokaryons between plasmid-transformed Chinese Hamster cells and yeast protoplasts were cultured in medium selecting for growth of yeast cells. The presence of the DNA sequence and its expression could be demonstrated in yeast-mammalian cell heterokaryons using Southern and Western blotting techniques (Ward, M. et al., Somat. Cell & Mol. Genet. 1986, in press). Ultrastructural studies of the fusion process have confirmed mixing of plant and animal cell cytoplasms (Davey et al., Protoplasma 96, 157, 1978) and that mixing is influenced by the fusion method. Although plant and animal cell organelles can be easily distinguished in heterokaryons soon after fusion, their ultimate fate is unclear. Mitochondrial recombination occurs frequently in plant protoplast heterokaryons, but rarely in animal cell hybrids. The use of organelle specific fluorescent dyes, such as Rhodamine derivatives for mitochondria, allied with fluorescence activated cell sorting, should facilitate the recovery of heterokaryon-enriched populations for further studies of cytoplasmically inherited traits in Inter-Kingdom fusion products.

60 SYSTEMATIC SHUT OFF OF hCG AND ACTH RECEPTORS AS OPPOSED TO THE EXPRESSION OF VIP, PGE_2 RECEPTORS AND OF THE STEROIDOGENIC ENZYMES IN LEYDIG ADRENAL INTRASPECIFIC HYBRIDS.[2] C. Finaz, A. Lefèvre, M. Vigier. INSERM/CNRS, Hôpital Debrousse, 69322 LYON, FRANCE.

When mouse adrenal and mouse Leydig cell genomes are facing each other in one cell, we observe a systematic shut off of their specific hormone receptors (ACTH and LH-hCG respectively). However, after more than 50 passages, one hybrid was able to reexpress ACTH receptors following a Robertsonian translocation with loss of chromosomal material : this result points out the role of a putative repressive gene that is lost in such an hybrid, and probably reflects what happens in the general process of cell differentiation. On the contrary, the two first steps of steroidogenesis which are common to both parental cells, i.e. adenylate cyclase and cyt. P450 C.S.C.C. activities, remained functional in 21 independent hybrid clones. We have further investigated the ability of 4 hybrid lines to express 1) the steroidogenic enzymes, 2) VIP and PGE_2 receptors.

Four hybrids were incubated with $-{}^3$H-progesterone which was principally converted to testosterone that represented 32 to 48 % of the total radioactivity in the medium at the end of the incubation period, and with $-{}^3$H-DHA that was mainly metabolized into testosterone (49 % of the total radioactivity) in one hybrid while, in the others, 3β-hydroxysteroid deshydrogenase activity was low. These results demonstrate that 3β-hydroxysteroid deshydrogenase, which is common to both parental cells, 20α-hydroxylase that is expressed only in the Y_1 cell line, 17α-hydroxylase, C_{17-20} lyase and 17α-oxydoreductase activities which are specific of the Leydig parent are expressed in the intraspecific hybrids. One hybrid responded to both VIP and PGE_2 stimulation by increasing respectively 4.1 and 4.9 fold its cAMP production, while the other two tested responded only to PGE_2 as the Leydig parent did : then the receptors to hormones that are not specific of the specialized parental cell function, i.e. PGE_2 and VIP, are not subjected to negative control and are still processed in the hybrids. Therefore, the negative control which reflects the antagonism between the specific parental functions in the hybrid genome acts specifically at the first step of the steroidogenic pathway : i.e. the gene(s) coding for hCG and ACTH receptor expression. This represents a simple and efficient genetic control of hormone target cell differentiation.

61 GENE TRANSFER IN HIGHER PLANTS. E.C. Cocking. Plant Genetic Manipulation Group, Department of Botany, University of Nottingham, Nottingham NG7 2RD, U.K.

In recent years, developments in plant cell biology have enabled new approaches to the study of gene transfer in plants. With further detailed study, the importance for agriculture and secondary product synthesis by plant cell cultures is likely to be profound. Central to these developments has been the availability of isolated plant protoplasts, including more recently the release of subprotoplasts from the apices of root hairs whilst still maintaining the structural integrity of the plant. This is likely to facilitate greatly the study of gene transfer and the interaction of plants with microorganisms (Cocking, Bio/Technology 3, 1104, 1985).

Regeneration of whole plants, from such isolated protoplasts, is possible from a steadily increasing number of dicotyledonous species, and a few monocotyledonous species, including recently rice protoplasts. The availability of such protoplast systems, lacking a cell wall, has enabled direct interaction of protoplasts with foreign DNA, and the production of transformed plants, in some instances at high frequency, is already well established; the likelihood of readily extending this approach to major crop plants, including rice, will be surveyed. Further developments in specific gene transfer will depend on having the 'gene in hand' (Pental and Cocking, Hereditas, 3, 83, 1985), and a better knowledge of the structure of plant genes and their developmental control.

Just as cell fusion has opened up new vistas in the study of gene transfer in animal cells, plant protoplast fusion, coupled with whole plant regeneration is opening up new vistas in gene transfer in plants, including both nuclear and cytoplasmic genes. Gene transfer between sexually isolated plant species has been established both within and between genera. Recently, for instance, fertile somatic hybrid plants of tomato (Lycoperiscon esculentum) and one of its wild relatives (L. peruvianum) have been produced by protoplast fusion; they were characterised by comparative morphology and Fraction 1

protein analysis. Chromosomally all the hybrids were hexaploids, and since backcrossing is possible these hybrids are likely to be of use in tomato improvement (Kinsara et al., Int. Jour. Plant Physiol. 1986, in press). This demonstration of gene transfer by somatic hybridisation opens up the possibility of incorporating novel traits from wild species into the cultivated tomato.

Gene flow between Nicotiana tabacum and Petunia hybrida has also been achieved. Leaf mesophyll protoplasts of a nitrate reductase deficient streptomycin resistant mutant of Nicotiana tabacum were fused with cell suspension protoplasts of wild type Petunia hybrida and selected cell lines were shown to be nuclear somatic hybrids. One of the cell lines regenerated cybrid plants which possessed the chloroplasts of N. tabacum in a predominantly P. hybrida nuclear background (Pental et al., Mol. Gen. Genet. 1986). Gene transfer by protoplast fusions can result in a range of novel combinations of chloroplasts and mitochondria in such cybrids, including mitochondrial recombinants and, at a very low frequency, chloroplast recombinants (Kumar and Cocking, Amer. J. Bot. 1986, in press).

Gene transfer in plants using these somatic cell procedures is often regarded as being divorced from gene transfer by sexual gametic fusion. However, recently it has been shown here, at Nottingham, that it is possible to fuse haploid protoplasts, isolated from pollen tetrads, with somatic leaf protoplasts. Gametosomatic hybrid plants were regenerated following the fusion of nitrate reductase deficient N. tabacum leaf protoplasts with N. glutinosa pollen tetrad protoplasts. These triploid interspecific gametosomatic hybrids were fertile on selfing and backcrossing, and their use could facilitate gene transfer between sexually isolated species (Pirrie and Power, Theoret. Appl. Genet. 1986, in press).

62 GENE TRANSFER IN FUNGI BY PROTOPLAST FUSION.

L. Ferenczy. Department of Microbiology, Attila József University, Szeged, P.O. Box 428, H-6701 Hungary

The wall of fungal cells can be removed by using enzyme complexes able to decompose its main macromolecular constituents. The resulting cells - protoplasts - are covered only by the cell membrane and are therefore suitable for genetic manipulations. Altered protoplasts too are able to regenerate new cell wall and develop colonies.

An overview is given of the recent advences in the following topics:

- Interspecific gene transfer: genetic analysis in asexual fungal species; elevation in ploidy level; gene transfer between cells of identical mating type; studies on cell cycle deficient mutants.
- Interspecific gene transfer: where are the limits?
- Separation of the nuclear and mitochondrial genomes with the protoplast technique; their selective and non-selective fusion transfer.
- Transformation of protoplasts with plasmids and isolated organelles.
- Transfer of nuclear genetic information by using inactivated protoplasts.

Though gene transfer via the protoplast fusion technique goes back only a relatively short period of time, it has become a powerful tool both for answering fundamental cytological questions and for solving practical problems of microbial strain improvement.

63 CELL TYPE SPECIFICITIES OF THE FRIEND AND MOLONEY MURINE LEUKEMIA VIRUS ENHANCERS.

Patrick Charnay, Hans-Jürgen Thiesen and Zsuzsa Bősze. Differentiation Programme, European Molecular Biology Laboratory, Postfach 10.2209, 6900 Heidelberg, Federal Republic of Germany.

The Moloney murine leukemia virus (Mo-MuLV) induces T cell lymphomas after injection into newborn NFS mice, whereas the Friend helper murine leukemia virus (Fr-MuLV) induces primarily erythroleukemia. The construction of recombinant viruses demonstrated the existence of a strong determinant of the distinct disease specifities of the two viruses within the U3 region of their long terminal repeats (LTR) (Chatis et al., (1983) Proc. Nat. Acad. Sci. USA 80, 4408-4411). Since this region contains the putative viral transcription control elements, including the transcriptional enhancer, these results suggested that the disease specificity might be determined, at least in part, by tissue-specific regulation of viral transcription. Such a direct relationship between tropism or disease specificity and cell type specifity of the viral enhancer has been established for several viruses.

These results led us to analyse the ability of the U3 region of the Fr-MuLV genome to promote transcription in a variety of human cell lines, including different types of haematopoietic cell lines. We find that the Fr-MuLV transcriptional control elements show striking preferential activity in erythroid cells. This preferential activity is due, at least in part, to the specificity of an enhancer located in the U3 region. This constitutes the first example of an enhancer with erythroid cell specificity. While no activity of the Fr-MuLV enhancer is detected in lymphoid cells, our data suggest that the Mo-MuLV enhancer is active in T-cells as well as in erythroid cells. Thus, the Mo-MuLV enhancers has a less restricted specificity than the Fr-MuLV enhancer and the different tissue selectivities and pathogenic properties of the two viruses may be explained in part by the different tissue specificities of their transcriptional enhancers.

64 Ooplasmic determinants during the remodelling of somatic cell nuclei in oocyte-thymocyte cell hybrids. D. Szöllösi(1), Renata Czołowska(2), Maria Sołtynska (3), A.K. Tarkowski(2). (1) I.N.R.A. Station de Physiologie animale, 78350 Jouy-en-Josas, France, (2) Department of Embryology, University of Warsaw, (3) Department of Cytology, University of Warsaw, 00-927/1 Warszawa, Poland.

Oocyte-thymocyte (Thy) cell hybrids were formed, using polyethylene glycole (PEG) as fusogene. Two different developmental pathways were followed depending on whether the oocytes were activated by 8% ethanol or not.

Nuclear envelope (NE) removing factor is active under both conditions when fusion takes place either with unactivated oocytes or close to the time of activation. Thy NE does remain intact, however, when fused five hours after activation.

Premature chromosome condensation (PCC) factor is active only in unactivated oocytes. The chromosomes condense to varying degrees even when originating from the same nucleus. The variation is more extensive when chromosomes from different nuclei in the same oocytes are compared.

Nuclear envelope reorganization occurs only when oocytes are activated. The NE may be constituted of the usual double membranes but frequently more layers (up to four) aggregate. In unactivated oocytes the chromosomes remain condensed during long culture after fusion.

Chromatin decondensation factor develops also only in activated oocytes, following reconstitution of the NE. Several nucleoli form which may fuse into one giant structure composed exclusively of thin filaments, a characteristic finding in pronuclei of mammalian zygotes. A slight chromatin decondensation occurs also when Thy nuclei are fused five hours after activation.

Thy nuclei entering into the cytoplasm of activated oocytes imitate closely the developmental pattern of sperm chromatin following normal fertilization (the experimental work carried out at the Dept. Embryology, University of Warszaw, was partially supported by WHO Small Supplies Programme).

65 NUCLEOCYTOPLASMIC INTERACTIONS AND GENE EXPRESSION IN HETEROKARYONS
N.R. Ringertz, Department of Medical Cell Genetics, Medical Nobel Institute, Karolinska Institutet, Box 60400, S-104 01 Stockholm, Sweden.

Heterokaryons made by fusing cells representing different species and forms of cell differentiation can be used to analyze the role of cytoplasmic factors in the regulation of transcription, replication and mitosis. Using fusions between nucleated chick erythrocytes (CE), which have repressed cell nuclei and transcriptionally active mammalian cells we have analyzed the role of nucleocytoplasmic protein exchange in the control of nuclear function. Intracellular migration of proteins has been studied with species specific antibodies to nuclear proteins and immunofluorescence methods.

Transcription and replication is reactivated in CE-nuclei introduced into rapidly growing mammalian cells. Early changes in CE-chromatin appear to be triggered by ionic signals. The growth of the chick nucleus is caused by a selective uptake of mammalian nuclear proteins. Among the proteins concentrated by the CE nucleus are mammalian RNA polymerases I and II, DNA polymerase alpha and non-histone nuclear proteins. If a transformed cell is used as the mammalian fusion partner, the CE nucleus can also be seen to concentrate oncogene coded nuclear proteins. Mammalian nuclear proteins are compartmentalized in the chick nucleus in structures homologous to those occupied by these proteins in mammalian nuclei. Mammalian RNA polymerase I is concentrated in the newly formed nucleolus of the CE-nucleus while mammalian nuclear envelope antigens appear in the chick nuclear envelope. Uptake of mammalian nuclear proteins appears to be an important event in the reactivation of the chick genome. Nuclear reactivation may, however, also in part be due to a loss of proteins from the inactive CE-chromatin. Thus H5 histone is lost from the CE-nucleus as it reactivates.

The pattern of chick gene activation is influenced by the choice of mammalian fusion partner. Chick globin genes are activated in some fusions but not in others.

66 COMPLETE SEQUENCE AND STRUCTURE OF A MAMMALIAN MYOSIN HEAVY CHAIN GENE: NO EVIDENCE FOR INTRON-DEPENDENT EVOLUTION OF THE ANCESTRAL MHC ROD GENE. Emanuel E. Strehler, Marie-Antoinette Strehler-Page and Bernardo Nadal-Ginard. Dept. Mol. & Cell. Cardiology, Children's Hospital Med. Ctr., Harvard Medical School, 300 Longwood Ave., Boston, MA 02115, U.S.A.

The locus corresponding to the rat embryonic skeletal muscle myosin heavy chain (MHC) gene has been obtained in three partially overlapping genomic clones. The complete nucleotide sequence of the MHC gene and of its immediate 5' and 3' flanking regions has been determined. The gene contains 41 exons distributed over 24 kb of DNA and encodes a 6035 nucleotide(nt)-long mRNA consisting of 90 nt of 5' untranslated, 5820 nt of protein-coding and 125 nt of 3' untranslated sequence. The MHC polypeptide is encoded by exons 3 to 41 and contains 1939 amino acids with a calculated Mr of 223,900. Its amino acid sequence displays the characteristic features of all sarcomeric MHCs, i.e., an aminoterminal "globular" head region and a carboxyterminal α-helical rod portion that shows the characteristics of a coiled coil with a superimposed 28-residue repeat pattern interrupted at only 4 positions by "skip" residues. The complex structure of the rat embryonic gene and the conservation of intron positions between this and other vertebrate and even invertebrate sarcomeric MHC genes suggests that they evolved from a highly split common ancestor. Introns in the rat embryonic gene interrupt the coding sequence at the boundaries separating the proteolytic subfragments of the head, but not at the head/rod junction or within stretches of highly conserved primary sequence that may correspond to functional domains. Significantly, not a single intron maps to a junction between two 28-residue repeats. Therefore, there is little evidence for exon shuffling and intron-dependent evolution by gene duplication as a mechanism for the generation of the ancestral MHC gene, particularly of its rod-encoding portion. Rather, intron insertion into a non-split ancestral MHC rod gene consisting of multiple tandemly arranged 28 residue-encoding repeats, or convergent evolution of an originally non-repetitive ancestral MHC rod gene must account for the observed structure of this part of present-day MHC genes.

67 PRIMARY STRUCTURE OF AN ALPHA-TUBULIN GENE OF PHYSARUM POLYCEPHALUM.

Mervyn J. Monteiro, Robert A. Cox. National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

An alpha-tubulin gene of Physarum was isolated as a phage λNM1149 recombinant (designated λNαTu). Phage λ NαTu contained a 4.7 kbp Hind III nuclear DNA fragment of the alt B locus of Physarum (one of four unlinked alpha-tubulin gene loci). Subfragments of the 4.7 kbp insert of phage λ NαTu were cloned into phage M13 and the nucleotide sequence was determined by the dideoxy chain termination method. The 4.7 kbp Hind III insert into phage λ NαTu spans the complete gene; sequences upstream of the 5'-end contain the RNA transcription promoter elements (the 'TATA' and 'CAAT' boxes). The nucleotide sequence encoding for alpha-tubulin contains seven intervening sequences, ranging from 62 to 200 nucleotides in size. The exons have a sequence that is identical with a Physarum alpha-tubulin cDNA clone (Krammer et al., J. Mol. Biol. 183, 633), except for three base-changes, one leading to a Val codon in place of a Met codon, another leading to a Glu codon in place of an Asp codon, and the third change is silent. The genomic clone provides the nucleotide sequence coding for the last 26 amino acids missing from the cDNA clone. The new sequence data indicates that the alpha-tubulin gene has a terminal methionine codon, and not a tyrosine codon which has been found in all alpha-tubulins sequenced to data. The nucleotide sequence data allows the construction of both oligonucleotide and antibody probes to examine the expression of the gene and the presence of the gene product not only during the mitotic cycle of macroplasmodia, but also throughout the life-cycle. A comparison of the expression of the gene in the plasmodial and amoebal phases is of particular interest.

68 THE MOLECULAR-CYTOLOGICAL ORGANIZATION OF THE TISSUE-SPECIFIC PUFF BRa IN CHIRONOMUS THUMMI. N.N.Kolesnikov, S.S.Bogachev, A.G.Blinov, V.M.Blinov, E.K.Gaidamakova, S.P.Fedorov, I.I.Kiknadze. Institute of Cytology and Genetics, Siberian Brench of Academy of Sciences, Novosibirsk, USSR.

Two clones hybridizing in situ with the tissue-specific puff BRa were identified in the microlibrary from the region A1-2 of C.thummi chromosome IV. The nucleotide sequence of two cloned fragments C1.2 and F6.2 (4,000 bp) was determined.These fragments contain two coding regions 720 and 738 bp long, respectively. These regions have 40% homologies and presumably represent genes or gene fragments. Their 5'-ends contain sequences (the TATA box and the capping site) for RNA polymerase II.The nucleotide sequences of the 5'-noncoding regions have definite homologies with the promoter of C.thummi globin gene. In both cases, the coding region has direct repeats 21 bp long at the ends containing the exon-intron sites. The repeats are 13- and 8-fold. The functional significance of these repeats requires futher investigation.

An element 597 bp long was identified in the fragment C1.2 after the direct repeats. This element is flanked by short direct target-repeats 5 bp long and repeats 107 bp long with different orientation. The inverted repeats contain the signal sequences for RNA polymerase II and the polyadenylation site. This element was reclоned. Hybridization in situ with the polytene chromosomes from different populations and species of Chironomus demonstrated that its localization varies in the range of 10 loci on chromosomes I-IV. Taken together, these characteristics of the sequence allow us to catogorise it as a mobile element of Chironomus genome.

69 UPTAKE OF NITROGEN-FIXING AZOTOBACTERS BY SOMATIC FUSION OF CELL-WALL MUTANTS OF CHLAMYDOMONAS REINHARDII. N.H.Nghia /2/, I.Gyurján /2/, P.Stefanovits /2/ I.Turtóczky /1/. /1/ Department of Genetics and /2/ Department of Plant Anatomy, Eötvös Loránd University, H-1088 Budapest, Muzeum krt. 4/a, Hungary.

The uptake of nitrogen-fixing Azotobacters into cells of eukaryotic green alga was induced by using polyethylene glycol /PEG/ treatment. Azotobacter cells were mixed with algal cells before somatic fusion of complementing arginine-requiring protoplasts of Chlamydomonas reinhardii. The algal protoplasts first aggregated and then fused in presence of bacterial cells. The electronmicrographs showed that the bacterial cells were taken up into the algal protoplasts after PEG treatment. In long-term experiments, the algal hybrids were obtained on selective medium, treated with lysozyme /final concentration : 8 mg/ml / and maintained on nitrogen and carbohydrate free medium for more than one year after the hybrids were observed. The cultures still grow and retain their green colour. The bacterial cells have been found in the intercellular space and inside some of the algal cells ten months after somatic fusion.

70 THE ELECTROFUSION OF SOMATIC CELLS OF TETRAHYMENA THERMOPHILA

Jacek Gaertig & Mauryla Kiersnowska. Institute of Zoology, Warsaw University Krakowskie Przedmieście 24/26, 00-927/1 Warszawa, POLAND.

For the first time, the electric field-mediated somatic fusion of a ciliate Tetrahymena thermophila is presented. Initially deciliated by dibucaine cells were brought into a close membrane contact by dielectrophoresis in a weakly conductive medium of 0,1 M sucrose in 1 MHZ sinusoidal field of 7,5 kV/cm intensity. Then the cell-fusion was induced by the application of repeated d.c. pulses of 50 μs duration and 12 kV/cm intensity. Up to 20 prestarved, logarythmic or stationary phase cells of the same mating type may form a single giant cell. Predominantly the cells fuse as linear arrays but some lateral fusion were also obtained. The cells may be joined in different mutual spatial configuration, either in homopolar or in heteropolar orientation and/or shifted by some angle. The polykaryons are fully able to regenerate cilia and became motile. After initial process of integration of fused components into a single syncytial organism the polykaryons gradually regulate into single cells and form viable clones with excellent efficiency. Thus electrofusion can be used as a research tool in studies on interaction of cells differing in their stages of clonal life, cell cycle, as a method of nuclear transplantation and in studies of the cell pattern regulation after variable fusions of the cytoskeletons.

71 CONSTRUCTION AND CHARACTERIZATION OF RECOMBINANT VACCINIA VIRUSES CARRYING HETEROLOGOUS VIRAL GENES. Istvan Fodor. Institute of Biochemistry and Physiology of Microorganisms, USSR Academy of Sciences, Pushchino, I42292, USSR

DNA segments of human hepatitis B and bovine leukemia viruses encoding antigens were ligated to a vaccinia virus transcriptional regulatory sequence by cloning in bacteria E.coli. The resultant recombinant plasmids were inserted into vaccinia virus genome by homologous recombination in vivo. Recombinant vaccinia virus strain carrying HBV sequences was capable of mediating the expression of HBsAg in infected mammalian cells. Infected rabbits produced antibodies to HBsAg. The expression of BLV gene inserted into vaccinia genome is being investigated. Other biological characteristics of the obtained recombinant viruses will be presented and discussed.

72 TELOMERIC-DNA SEQUENCES DIFFERENTIALLY ACTIVATED BY HEAT-SHOCK IN TWO CHIRONOMUS SPECIES.

G. Morcillo(1), M.J. Carmona, T. Carretero and J.L. Díez. Centro de Investigaciones Biológicas. Velázquez, 144. Madrid-28006. (1) Universidad Nacional de Educación a Distancia. Madrid-28040. Spain.

Chironomus thummi shows the typical heat-shock response reported in many eukaryotic systems. However, a peculiarity of this system is the formation of a Balbiani ring at some telomeric regions, a phenomenon that we are currently investigating. We have cloned and sequenced a DNA fragment from a heat-shock induced Balbiani ring at the right telomere of C. th. thummi chromosome III. Southern analysis of genomic DNA showed that the cloned fragment represents a basic repeating unit clustered in tandem arrays, being around 6.900 copies in the genome. Hybridization to chromosomal DNA evidenced homologous sequences at all the telomeres except the kinetochoric and of chromosome IV. They were also present at some interstitial sites. However, under different heat-shock conditions, a puff was always formed at the right telomere of chromosome III transcribing an RNA complementary to the cloned fragment. On the contrary other regions sharing homologous DNA sequences were only occasionally or never activated. We have searched for homologous sequences in other species. Cross-hybridization was only found in the related subspecies C. th. piger. The sequence had the same telomeric distribution as in C. th. thummi. But, in this case, heat shock did not induced the activation of the right telomere of chromosome III. Instead, a Balbiani ring was formed at the telomere of chromosome IV. This was confirmed in thummi x piger hybrids. The sequences shared by all the telomeres and common to the two subspecies are differentially activated by heat-shock.

73 CARBON DIOXIDE TREATMENTS INDUCE THE HEAT-SHOCK RESPONSE IN CHIRONOMUS THUMMI.

D. Barettino[1,2], G. Morcillo[1,2] and J.L. Diez[1]. (1) Centro de Investigaciones Biológicas (CSIC), Velázquez, 144, 28006-MADRID. (2) Facultad de Ciencias. UNED, 28003-Madrid (Spain).

The characterization of the heat-shock response in the salivary gland cells of Chironomus thummi has shown that the basic features of this phenomenon, namely the induction of specific puffs and the synthesis of heat-shock polypeptides (hsp's), are essentially similar to those reported in Drosophila. However, we have found striking differences in relation to the induction of this response by agents other than heat-shock. Physical treatments, as anoxia, and drugs, as Arsenite, Dinitrophenol, Hidrocortisone, benzamide or Cloramphenicol, that induce, totally or partially the heat-shock response in Drosophila, failed in Ch. thummi. Among the treatments tested, only carbon dioxide, whose effects are not well characterized in Drosophila cells, induces the heat-shock response in C. thummi larvae. This response can be visualized during the recovery of 60-120 min treatments with carbon dioxide. All the heat-shock puffs previously described are induced, including the telomeric Balbiani ring T-BR-III. Besides, some puffs not heat inducible are also present. The transcriptional activity of T-BR-III has been also analyzed by DNA/RNA "in situ" hybridization with the cloned sequence λ-Cth-5, that is transcribed during heat-shock.

Protein synthesis in the salivary gland cells are also modified, showing the typical pattern of the heat-shock polypeptides. However, the induction of this polypeptides takes place in a non-coordinate way.

74 HEAT SHOCK RESPONSE IN PLANTS AND THERMOPROTECTION.

Nelson Marmiroli (1), Miriamo Odoardi Stanca (2), Francesca Tassi (1), Francesco Restivo (1), Valeria Terzi (1), Mariangela Ardiani (1), Elena Maestri (1), Natale di Fonzo (3), Carlo Lorenzoni (4). (1) Institute of Genetics University of Parma - Parma, (2) Experimental Institute for Cereal crop - Fiorenzuola D'Arda, (3) Experimental Institute for Cereal crop - Bergamo, (4) Institute for Plant Genetics - Catholic University - Piacenza.

Transfer from normal temperatures (20-25°C) to high temperatures (40-45°C) determins in barley plantlets the appearance of a new set of proteins which have been termed heat shock proteins (HSP). By comparing heat shock response in two barley cultivars with different growth habit and different heat and cold tolerance (cv. Georgie - cv. Onice) we have shown some differences in the HSP synthesized by the two cultivars. Te differences have been confirmed also by "in vitro" translation of the mRNA isolated from the two cultivars after the heat shock. Southern analysis with restriction fragments from the clone 229 containing the cloned gene for the 70 kD protein of Drosophila, evidenced the presence of homologous sequences within the barley genome. Other barley heat shock genes are under investigation by Southern analysis with probes containing the sequences for other Drosophila heat shock genes. The role of HSP in acclimation and thermoprotection has been analyzed in plant subjected to different temperature increases. Some of the HSP recognized have been localized within the chloroplasts and studies have been undestaken to see wether there are HSP synthesized within these organelles.

75 HORMONAL REGULATION OF RABBIT MAMMARY mRNA METABOLISM

P.J. Tighe (1), C.J. Wilde (2), R.J. Mayer (1). (1) Department of Biochemistry,University of Nottingham, Clifton Boulevard, Nottingham, England. NG7 2UH . (2) Department of Lactational Physiology and Biochemistry, Hannah Research Institute, Ayr, Scotland. KA6 5HL

A cDNA library was generated from mRNA isolated from the lactating mammary gland of White New Zealand rabbits. The cDNAs were inserted into the Pst I site of plasmid pBR322 by C-tailing. The resultant clones were then differentially screened by probing with either alpha and beta casein cDNAs (pRcas 928, pRcas 924, supplied by J.P. Kraehenbuhl, Institute of Biochemistry, University of Lausanne, Lausanne, Switzerland.) or a single stranded cDNA probe produced from total lactating gland mRNA by reverse transcription. colonies which hybridized positively to the total mRNA derived probe alone were studied further. Plasmid DNA was prepared from these clones, radiolabeled and used to probe northern blots of total RNA from mid-pregnant and mid-lactating rabbit mammary gland. cDNAs which showed enhanced hybridization to a specific mRNA in lactating gland were further characterized by hybrid selection of the mRNA and translation in vitro in a rabbit reticulocyte lysate system, in the presence of [35-S] methionine. Translated polypeptides were identified by electrophoresis on SDS-PAGE gels according to Laemmli. Five clones which hybridize to separate mRNAs are being used to measure the accumulation and stability of the specific mRNAs during pregnancy and lactantion, using RNA isolated from animals throughout this period, by both DNA and RNA excess hybridizations on solid supports.

76 ESTROGEN INDUCTION OF A SPECIFIC NUCLEAR PROTEIN IN LIZARD OVIDUCT.

Gaetano Ciarcia(1),Virgilio Botte(2),Massimo Lancieri(3),Hisanori Suzuki(4). (1)Dipartimento di Biologia evolutiva e comparata;(2)Istituto e Museo di Zoologia;(3)Dipartimento di Genetica,biologia generale e molecolare;(4)Dipartimento di Chimica organica e biologica.Università di Napoli -Italia -

The development of lizard Podarcis s.sicula oviduct depends on the function of the ovarian sex hormones.In spayed females,infact,this organ becomes atresis and 17β-estradiol can restore biochemical and morphological modification which match the oviduct recovery and maturation.Biochemical analysis of nuclear oviductal proteins shows that peculiar modifications happen in histone-rich fractions which are correlated to oviduct maturity.When this histone-rich fraction is subjected to electrophoresis in the presence of urea-acetic acid and SDS-PAGE,it can be found a protein band with a higher mobility than H_4. This protein is not detectable in spayed females but its appearance can be induced by 17β-estradiol administration.No trace of the protein can be observed in oviductal cytoplasmic fractions or in nuclear and cytoplasmic fractions of other organs.The molecular weight of the protein was estimated to be 9.9kDa by SDS-PAGE.The aminoacid composition,however,is quite different from that of histones,from that of the so-called high mobility group and that of ubiquitin. The aminoacid composition of this protein,the limited period of its appearance and its impressive amount raise pose questions on its physiological role in the nucleus.One of the hypothesis might a possible implication in the controll of gene expression at a very particular time of the sexual cycle.

77 EFFECTS OF ACTH ON ^{3}H-URIDINE INCORPORATION IN ADRENAL CELLS, IN VITRO.AUTORADIOGRAPHIC STUDY.

Maria C.Magalhães (1), A.Bonito-Vitor (1) and M.M.Magalhães (1) - Institute of Histology and Embryology of the Faculty of Medicine of Oporto and Center of Experimental Morphology of the University of Oporto, 4200 Porto, Portugal.

Adrenal slices from eight male rats ~35g in weight, were incubated in Krebs-Ringer-bicarbonate solution with glucose, at 37°C, containing 5,6-^{3}H uridine (100 μCi/ml). After 30 min some slices were removed from the medium and fixed in glutaraldehyde and osmium tetroxide; the remaining were transferred to the incubation medium with "cold" uridine (100 μg/ml) and fixed at 15, 30, 45 and 60 min (Group I). Slices from another 8 animals with the same weight were incubated in the same medium containing 100 mU/ml of ACTH (Group II). Pieces were washed during 72 h, embedded in Epon 812, and processed for light and ultrastructural radioautography. Semithin sections, 1 μm thick, of both Group I and II and of similar incubation times were radioautographed on the same slide,and exposed during 3 and 7 days.Ultrathin sections were developed in Kodak D19b and Elon-ascorbic acid (EAS), and exposed during 45 and 70 days.

At 30 min,nuclear labelling of Group I animals was stronger than in Group II;grain counting confirmed these findings (101,01 vs 11,31 silver grains/1000 μm^2). At the other incubation times the autoradiographic reactions were always more intense in Group I than in Group II. Qualitative electron microscopic radioautographs analysis showed that in Group I animals the autoradiographic reactions were localized over the chromatin and nucleolus. In Group II animals nuclear labelling was scarce.

These results show that ACTH has a precocious inhibitor effect on the RNA synthesis in the adrenal which confirm in vivo experiments.

78 EFFECTS OF TRIIODOTHYRONINE (T_3) ON THE DEVELOPMENT OF SOME NEURONAL MARKER-PROPERTIES AND ON EXPRESSION OF GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP) IMMUNOREACTIVITY IN PRIMARY BRAIN CELL CULTURES.

E. Madarász and T. Vitray Jr. Dept. of Comparative Physiology, Eötvös Loránd University, Budapest. H-1088. Múzeum krt 4/A, Hungary

Influences exerted by triiodothyronine on in vitro development of neural cells were studied in primary cell cultures obtained by mechanical dissociation from embryonal (E_{17}) rat forebrains. Serum-containing cultures were maintained at various concentrations (0-20 nM) of T_3 for 3 to 4 weeks after plating. Light microscopic observations on cell survival and on morphological development were statistically analysed. Cell proliferation was investigated by measuring ^{3}H-thymidine incorporation and by visualization of proliferating cells by autoradiography. In order to study neuronal marker - properties, choline-acetyltransferase activity was assayed radioenzymatically, and GABA immunocytochemistry was used to follow the development of GABA ergic neurons. The differentiation of astroglial cells in control and hormon treated cultures was investigated by GFAP immunocytochemistry qualitatively, and enzyme linked immunosorbent assays were carried out for semi-quantitative evaluation of GFAP containt of the cultures. Data obtained by various methods showed that T_3 enhances the expression of all of the neuronal marker-properties investigated, while GFAP immunoreactivity was reduced as a consequence of hormone treatment. Possible intercations between the glial proliferation and neuronal differentiation are discussed in relation to effects of T_3 exerted on differentiation of neural tissue.

79 SYNTHESIS OF A TRANSLATIONALLY CONTROLLED 23-KD PROTEIN DEPENDS ON THE RATE OF PROLIFERATION OF EHRLICH MOUSE ASCITES CELLS

Peter Nürnberg, Rainer Benndorf, Heinz Bielka. Central Institute of Molecular Biology, Department of Cell Physiology, Academy of Sciences of GDR, DDR-1115 Berlin-Buch, Robert-Rössle-Str. 10, German Democratic Republic

For analysis of newly synthesized proteins tumor cells grown in the peritoneal cavity were transferred to culture medium containing ^{35}S-methionine. After labeling the cells were processed for 1- and 2-dimensional electrophoresis. Studying tumor cells of different age (corresponding to different proliferation rates) one of the major alterations concerns protein p23 (pI 5.0) whose synthesis declines with increasing age of the tumor. Addition of serum or insulin to serum depleted cells induces several proteins including p23 resulting in a protein pattern characteristic for cells of fast proliferating tumors. The extent of p23-induction reaches its maximum between the 7th and 10th day of tumor growth. Epidermal growth factor and other effectors of cell growth tested so far did not induce p23. In contrast to other cell proteins, p23 was also induced in the presence of cordycepin and α-amanitin. Analyzing the time course, the induction of p23 could be detected already 10 min after serum addition. The latter findings strongly indicate a translationally controlled process. Surprisingly, actinomycin D alone is also sufficient to induce p23 with the same short-time kinetics, resembling other reported effects of this drug previously termed "superinduction".

80 IMMUNOLOGICAL EVIDENCE FOR THE EXISTENCE OF H1-LIKE HISTONE IN YEAST.

Ljuba Srebreva (1), Jordanka Zlatanova (2). (1) Institute of Molecular Biology, Bulgarian Academy of Sciences, 1113 Sofia, and (2) Institute of Genetics, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

In view of the controversies about the existence of histone H1 in yeast we have reinvestigated the problem by studying perchloric acid- and salt-extracted yeast proteins.

Perchloric acid-extracted proteins from whole cells contain only two fractions which comigrate with "authentic" yeast HMGs both in SDS- and in acid-urea gels. These extracts show a considerable cross-reaction with anti-calf thymus HMG-antiserum and do not react with antiserum to mouse liver H1.

The isolation of "authentic" yeast HMG by the standard salt-trichloroacetic acid procedure gives two types of preparations containing different number of protein bands. The preparation poorer in proteins reacts only with the anti-HMG-antiserum whereas the richer preparation gives also considerable cross-reaction with the anti-H1-antiserum. Immunoblotting analysis performed on the salt-extracted proteins reveals the presence of three protein bands giving positive immunoreaction with the anti-H1-antiserum. Two of these possess electrophoretic mobilities close to that of the marker calf thymus H1 and similar to the mobilities of presumptive yeast H1 fractions found by other authors. The third fraction probably represents an H1-dimer as suggested by its characteristic electrophoretic mobility in SDS-containing gels.

81 BINDING OF EPSTEIN-BARR VIRUS (EBV) NUCLEAR ANTIGENS (EBNA) TO EBV DNA.

Josef Reischig (1), Ivan Hirsch (2), Dušan Bartsch(2). (1) Faculty of Medicine, Charles University, Pilsen, and (2) Institute of Sera and Vaccines, Praque, Czechoslovakia.

Epstein-Barr virus (EBV) nuclear antigens (EBNA) were purified from Raji cells on DNA-Sepharose, Blue-Sepharose and hydroxylapatite columns and characterized by acid-fixed nuclei binding technique (AFNB) and by SDS-polyacrylamide gel electrophoresis. The binding of resulting antigens to intact virion EBV DNA (B95-8) and to individual fragments of overlapping cosmid library of RBV DNA (M-ABA) was visualized by electron microscopy using several methods of specimen preparation and contrasting. In some experiments there was utilized specific reaction of EBNA-1 with rabbit antibodies raised against the part of EBNA-1 encoded by IR3 area of EBV DNA. We have found that EBNA-containing DNA-binding proteins bound to several sites of EBV DNA molecule forming often heterogeneous complexes. The areas of the genome with the maximum frequency of binding of protein molecules were mapped by computer analysis. In agreement with the regulatory function of EBNA the binding reveals a high degree of topological specification.

We thank Dr. G.W.Bornkamm for the gift of EBV (M-ABA) cosmid clones and Dr.E.Kieff for EBNA-1 specific antibodies.

82 TERATOCARCINOMA STEM CELL MUTANTS DEFECTIVE IN THE EXPRESSION OF EMBRYONIC ANTIGENS TEC-1 AND TEC-1,2,3. Petr Dráber, Petr Malý. Institute of Molecular Genetics, Cz. Acad. Sci., Vídeňská 1083, 142 20 Prague 4, Czechoslovakia

Mouse embryonic antigens TEC-1,2,3 are glycoconjugates that are expressed on mouse preimplantation stage embryos and teratocarcinoma stem cells (TSC) but not on most adult tissues and cells. To study the regulation of the expression of TEC-1,2,3 antigens and to determine their importance to the course of differentiation, mutants lacking TEC-1 or TEC-1,2,3 antigens were isolated from TSC lines P19-X1 and P19S1801A1 Monoclonal antibody TEC-01 conjugated to cytotoxic plant lectin ricin (TEC-01-RIC) was used as selective agent. Eleven independt subclones that are resistant to the cytotoxic effect of TEC-01-RIC were isolated in single step selection procedure. Two TEC-1$^-$ mutants of P19S1801A1 and one TEC-1$^-$,2$^-$,3$^-$ mutant of P19-X1 cells were characterized in detail. Somatic cell hybrids constructed between TEC-1$^-$ and TEC-1$^+$ cells expressed parental amount of TEC-1 antigen suggesting that the mutant phenotypes are recessive. Hybrids between TEC-1$^-$ cell lines were TEC-1$^+$ indicating that at least two complementation groups were found. A 2D gel electrophoresis did not show any reproducible differences among mutant and parental cells. However, when extracts from parental and mutant cell lines were separated by SDS-PAGE and immunolabeled with TEC-01, only parental cells exhibited broad antigenic peak of MW>200 000. The mutants may be useful for studying biosynthesis and developmental aspects of developmentally regulated surface glycoconjugates.

83 CONTROL OF ALPHA-FOETOPROTEIN GENE EXPRESSION: ANALYSIS OF HEPATIC CELL HYBRIDS.
Josiane Szpirer & Claude Szpirer. Departement de Biologie Moléculaire, Université Libre de Bruxelles, B1640 Rhode-St-Genèse, Belgium

Mouse hepatoma cells of a clonal line, which produce albumin and alpha-foetoprotein (AFP) were fused with the following cells:
-rat fibroblasts, which do not produce albumin and AFP
-adult rat hepatocytes, which produce albumin but not AFP
-foetal rat hepatocytes, which produce both albumin and AFP.
Hybrids were selected from each of these fusions and tested for production of both mouse and rat albumin and AFP. It was observed that the rat albumin gene can be activated in some hybrids of the first fusion and was always maintained in the active state (when present) in the hybrids of the two other fusions. On the contrary, the AFP gene was never activated and almost never retained in the active state, in any of the hybrids isolated, although the mouse AFP gene is expressed in the analyzed hybrids. The presence of a silent rat AFP gene was demonstrated by Southern blot analyses using a rat AFP probe. These experiments show that, unlike the albumin gene, a normal rat AFP gene is inactive or inactivated, in a mouse hepatoma environment and the relevance of this observation will be discussed.

84 A COMPARATIVE STUDY OF THE BALBIANI RINGS (BR) AND THE SECRETORY FUNCTION OF THE SALIVARY GLANDS IN GENUS CHIRONOMUS.
I.I. Kiknadze, T.E. Sebeleva, A.G. Istomina, T.M. Panova, N.N. Koiesnikov. Institute of Cytology and Genetics of the USSR Academy of Sciences Siberian Department, 630090 Novosibirsk USSR.

In C.thummi, 3 BRs (BRa, BRb, BRc) contain genes for the synthesis of the tissue-specific secretory proteins. The secretory proteins were analyzed electrophoretically in the larvae exposed to galactose for their whole life (0.1-1.0 μg/ml). From the results it was concluded that BRc and BRb code for the high molecular fractions sp1a and sp1b, respectively. The additional BRa, which develops only in the 4 cells of special lobe, was found to code for the smaller fraction sp160-180 kD. Different sizes of m-RNP particles were distinguished in BRs in accordance with the sizes of the secretory proteins coded: BRc and BRb from RNP particles with d-40.2-41.4 nm, BRc gives rise to RNP particles with d-29.7 nm. Localization and sizes of chromomeres forming the BRs were determined using DNA clones (pCthBRaF6, pCthBRaC1, pCthBRc16, pCthBRb19). Taking the above characteristics of the C.thummi BRs and the secretory proteins of the secretory proteins of the salivary gland as standard, we carried out a comparative study of 20 species of the genus Chironomus. It was found that besides C.piger, the most closely related to C.thummi are the species members of pseudothummi complex. In the species of the thummi complex (C.plumosus, C.balatonicus et al.) the BRs differ in sizes of the RNP particles (d-34.0-34.8 nm in BR1, BR3, BR4 and 38.8 nm in BR2); cross hybridization of DNA clones derived from C.thummi BRs is more difficult; the special lobe secretion cannot be identified.

85 CASEIN cDNA TRANSFECTION INTO MAMMARY CELLS USING A BOVINE PAPILLOMA VIRUS VECTOR.
Tom Burdon (1), John Windass (2), R. John Mayer (1). (1) Department of Biochemistry, University of Nottingham, Nottingham NG7 2UH, U.K, and (2) ICI Corporate Biosciences, The Heath, Runcorn, U.K.

With a view to studying the mechanisms of milk gene expression and milk protein secretion, expression vectors containing the rabbit α and β casein cDNAs (from Dr. J-P. Krahenbuhl, Lausanne, Switzerland) have been made. These constructs contain two elements, a plasmid pdMMT (341-1) and the complete Bovine papilloma virus genome (BPV-1) (from Dr. P. Howley, Bethesda, U.S.A.).

The plasmid pdMMT consists of a deletion derivative of pBR322, pML2 which lacks the so called poison sequences plus a transcriptional cassette containing a mouse metallothionein promotor and the SV40 small 't' antigen splice and termination signals separated by a clonable BgLII site. Full length cDNAs for both the α and β caseins have been inserted at this unique site using adaptor CL59 to ligate the PstI released cDNA to the BgLII cohesive ends. Plasmids pdMCα and pdMCβ were cloned into the unique BamHI site of BPV-1 producing the vectors pBMCβ(1,2) and pBMCα(5,6).

We are currently transfecting mouse (C127) and rabbit mammary cells with these vectors to study expression of the casein cDNAs.

86 A COMPARATIVE STUDY OF REGULATION OF TRANSFECTED HUMAN AND ENDOGENOUS MOUSE INTERFERON GENES IN LMTK$^-$ CELLS
Miklos Toth, Ojunsuren Cendsuren*, Valeria Endresz$^+$ and Erno Duda
Institute of Biochemistry, Biological Research Center, Szeged, H-6701, Institute of Microbiology,$^+$ Medical University of Szeged, H-6720, Hungary, and Institute of Experimental and General Biology,* Ulan-Bator-51, Mongolia

Expression and regulation of transforming foreign genes and corresponding host genes are investigated in tissue cultured mammalian cells. Human interferon-α_1 gene with its flanking regions was introduced into mouse LMTK$^-$ cells by cotransfection with selectable marker genes (neo^r of Tn5). Transcriptional regulation of the foreign gene seemed to be regulated like the endogenous equivalent, since clones of genetically transformed cells produced biologically active human interferon in parallel with mouse interferon upon Sendai virus induction. On the other hand, only mouse interferon synthesis was a subject of "priming", that is enhanced by pretreatment of cells with a low amount of interferon, prior to induction. It means, that regulation of transformed human interferon sythesis was not identical to that of the endogenous mouse interferon protein. Obviously the "priming" mechanism (its molecular meaning is not known) is able to distinguish endogenous and transforming genes.

Inducible expression of human IFN gene was diminished gradually in transformed cells during serial passage of cells. Possible mechanisms involved in the inactivation of transformed human interferon gene are discussed.

87 A TRANSCRIPTIONAL ENHANCER WITH SPECIFICITY FOR ERYTHROID CELLS IS LOCATED IN THE LONG TERMINAL REPEAT OF THE FRIEND MURINE LEUKEMIA VIRUS. Zsuzsa Bösze, Hans-Jürgen Thiesen, Patrick Charnay. Differentiation Programme, European Molecular Biology Laboratory, Postfach 10.2209, 6900 Heidelberg, Federal Republic of Germany.

We made use of the chloramphenicol acetyl transferase (CAT) system to investigate the ability of the U3 region of the long terminal repeats (LTR) of the Friend murine leukemia virus (Fr-MuLV) and Moloney murine leukemia virus (Mo-MuLV) to promote transcription. The analysis of the expression of Fr-MuLV-CAT constructs in a variety of human cell lines reveals the presence, in the U3 region, of a transcriptional enhancer with specificity for erythroid cells. This constitutes the first example of an enhancer with such a property. Analysis of the Mo-MuLV enhancer suggests that it is active at least in erythroid and lymphoid cells and has thus a less restricted specificity than the Fr-MuLV enhancer. The different tissue specificities of the two enhancers correlate with the different tissue selectivities and pathogenic properties of the two viruses.

88 EFFECT OF CO-INJECTED PROTEIN EXTRACT FRACTIONS ON THE TRANSCRIPTION OF CLONED XENOPUS AND MOUSE RDNA FRAGMENTS AFTER INJECTION INTO XENOPUS LAEVIS OOCYTES.
Ansgar Hofmann(1), Ingrid Grummt(2), Michael F. Trendelenburg(1). (1) Institute of Cell and Tumor Biology, Institute of Experimental Pathology, German Cancer Research Center, D-6900 Heidelberg, FRG, and (2) Institute of Biochemistry, University of Würzburg, D-8700 Würzburg, FRG.

Injection of cloned DNA fragments into Xenopus laevis oocytes is one of the most favourable systems to study transcriptional events in a in vivo situation. It is possible to analyse some of the parameters by means of electron microscopic spread preparation as well as by the S1-mapping technique.

Special Xenopus laevis rDNA constructs were made in order to discriminate transcripts derived from injected rDNA against endogenous transcripts. The influence of deletions in the 3'-region on the process of transcription termination was investigated in detail. In addition the influence of co-injected homologous and heterologous (mouse) protein extracts on transcription efficiency of these templates was analysed. Striking differences were observed, dependant on the experimental protocol used: i) injection of the extract into the cytoplasm, ii) injection of the extract into the nucleus, iii) preincubation of the DNA with the extract.

For transcriptional studies on mouse rDNA a construct, consisting of 5'- and 3'-sequences of the mouse rRNA gene, was used for injection. By contrast to the homologous Xenopus rDNA system, transcription of injected mouse rDNA is in this heterologous system below the detection level. Complementation with subfractions of mouse cell extracts stimulates the transcription to higher levels.

89 PEPTIDE FACTORS INVOLVED IN REGULATION OF TRANSCRIPTION IN TETRAHYMENA.
H. A. Andersen and Henrik Islin. Department of Biochemistry B, Panum Institute, University of Copenhagen, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark.

An exponentially multiplying population of the protozoa Tetrahymena has highly variable transcription rate when cultivated in a complex broth medium. It has been shown that peptides in the medium specifically stimulate transcription and that the cells in response to this stimulation synthesize a peptide with a molecular weight of around 4.000 D. This peptide inhibits transcription in vivo in cells with high rate of transcription and in in vitro transcription systems. We have purified the transcription inhibitor and found that when cells are stimulated to high transcription rate with stimulatory peptides they selectively accumulate inhibitor peptide in the nuclei. In vitro experiments in which isolated nuclei were incubated in the presence of the inhibitor peptide have shown that the inhibitor reduces the RNA polymerase activity in the nuclei by binding to the polymerase itself. The RNA polymerase-peptide complex has lower affinity for chromatin and is released during the incubation of the isolated nuclei. On the other hand stimulatory peptides will compete with the inhibitor in binding to the RNA polymerase and the polymerase activity in isolated nuclei is determined -at least partially- by the ratio between stimulatory and inhibitory peptide factors. It is suggested that regulatory peptides also in vivo are involved in regulation of transcription by determining the activity of the RNA polymerase.

90 THE ACTIVITY OF RNA POLYMERASE II IN VIVO IS REPRESSED WHEN PHOSPHORYLATION OF A 42 kDa NUCLEAR PROTEIN IS COMPETED OUT BY MICROINJECTED PHOSVITIN.
Endre Egyházi, Mikael Holst, Andrew Pigon. Department of Histology, Karolinska Institutet, S-104 01 Stockholm, Sweden

The findings that chromatin proteins, especially those engaged in the transcriptional machinary, are phosphoproteins led to the suggestion that postsynthetic protein phosphorylation possesses a regulatory potential. We have found that the transcriptionally active chromatin sites are highly enriched in rapidly phosphorylated 25 kDa, 30 kDa, 33 kDa and 42 kDa polypeptides. We have in the present work used a microinjection technique to examine whether injected phosvitin, an efficient substrate for casein kinase NII, could compete out the endogeneous phosphorylation of any of these nuclear proteins and thereby interfering with the activity of RNA polymerase II. Phosvitin transferred into nuclei of salivary gland cells becomes phosphorylated by the endogeneous nuclear protein kinase(s). Phosphorylation which utilizes ATP as phosphate donor was separated from phosphorylation which uses GTP for phosphate transfer. The egg yolk protein incorporates phosphates from ATP as well as from GTP and the phosphorylation is heparin-sensitive indicating that phosvitin is phosphorylated by casein kinase NII. Microinjected phosvitin does not seem to affect the endogeneous incorporation of phosphate groups from ATP into nuclear proteins but protein phosphorylation by GTP is interrupted. Apart from a minor overall reduction of ^{32}P incorporation, the phosphorylation of a 42 kDa nuclear protein, a putative transcription stimulatory factor, was competed out by more than 80 % compared to the control value obtained in the absence of phosvitin. Parallel analyses of DNA transcription in phosvitin injected nuclei showed that the RNA polymerase II-mediated synthesis of hnRNA and Balbiani ring RNA was diminished by 80 % and 90 %, respectively. By contrast, the transcription of nucleolar preribosomal 38 S RNA by RNA polymerase I remained unaffected. The results taken together suggest a causal relationship between the modification of the GTP-dependent phosphorylation of a 42 kDa protein and the activity of RNA polymerase II.

91 REGULATION OF rDNA TRANSCRIPTION IN CHO CELLS. F. Amalric, G. Bouche, B. Bugler, M.Caizergues-Ferrer, B. Lapeyre and M. Mathieu, Centre de Recherche de Biochimie et de Génétique Cellulaires du C.N.R.S., 118 route de Narbonne, 31062 Toulouse cedex, France.

In higher eukaryotic cells, ribosomal genes (100 to 300) are localized in a particular structure, the nucleolus. They are highly transcribed in exponentially growing cells and in resting cells, their expression can be modulated by various stimuli. "In vivo" several factors, in particular short lived proteins, have been proposed to play a role in the regulation of pre rRNA synthesis. "In vitro",nucleolin, the major phosphorylated nucleolar protein in exponentially growing cells, acts in run off experiments as an inhibitor of rRNA transcription in presence of a protease inhibitor, leupeptin, while in absence of the inhibitor, nucleolin is cleaved in specific species and rDNA transcription is unaffected. The pattern of nucleolin cleavage is directly dependent on phosphorylation level of the molecule.

An increase in phosphorylation results in an increase of maturation. A cyclic AMP independent nucleolar protein kinase (NuII like) was shown to specifically phosphorylate nucleolin. The kinase activity is stimulated by spermine. Several of the factors shown "in vivo" to stimulate pre rRNA synthesis, produce an increase in spermine concentration and thus act on nucleolin phosphorylation. In particular ornithine decarboxylase, a short lived enzyme involved in spermine biosynthesis, has been proposed to regulate directly pre rRNA transcription.

Taking into account our results on nucleolin functions (interactions with rDNA, nucleolar substructures, rDNA transcription) and factors that were shown to act on pre rRNA synthesis "in vivo", we propose a model of regulation of pre rRNA synthesis in which the key steps would be the phosphorylation of nucleolin by a type NuII kinase.

92 APOLIPOPROTEIN A-I EXPRESSION IN VARIOUS CHICKEN TISSUES AT DIFFERENT STAGES OF DEVELOPMENT. Stefano Ferrari(1), Maria Capuano(1), Enoe Drusiani(1), Patrizia Tarugi(2), Sebastiano Calandra(2), Marina Fregni(1). Institutes of (1)Biological Chemistry and (2)General Pathology, University of Modena, Via Campi 287, 41100 Modena, Italy.

Apolipoprotein A-I (Apo A-I), which represents the major protein component of serum high density lipoproteins, is mainly expressed in the small intestine and liver of the adult chick. However the isolation of a cDNA clone representing the entire sequence of Apo A-I mRNA and its use as a hybridization probe has allowed us to demonstrate that Apo A-I mRNA is transcribed from a single copy gene even in the brain, heart and skeletal muscle of the adult animal (the amount of Apo A-I mRNA in these organs is respectively 18%, 13% and 11% of the amount present in the intestine, arbitrarily taken as 100%). Furthermore, it was possible to observe, by Northern blot hybridization experiments, that the tissue specific expression of Apo A-I mRNA changes dramatically around hatching. The liver appears by far the major site of Apo A-I mRNA production between days -7 and +2 (taking hatching as day 0). Contrarywise the concentration of Apo A-I mRNA in the intestinal epithelium is very low until hatching, it increases steeply between day +2 and +6 and it levels off between day +6 and +10, the small intestine representing from that time on the major site of Apo A-I mRNA production. Quite interestingly the breast muscle appears to express considerable amounts of Apo A-I mRNA only in the very limited period between day 0 and +2 (about 50% of the amount present in the liver at the same time). Finally brain and heart do not show any significant variation in their Apo A-I mRNA content throughout the investigated time span. We suggest that development has a remarkable effect on the tissue specific expression of Apo A-I mRNA.

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93 A NEW FAMILY OF REPEATED SEQUENCES IN MOUSE GENOME. Marta Di Carlo* and Marialuisa Melli°. *Istituto di Biologia dello Sviluppo C.N.R., via Archirafi 20, 90123 Palermo, Italy and °Istituto SCLAVO, centro ricerche, via Fiorentina 1,53100 Siena, Italy.

A clone with a repeated sequence has been isolated from a cDNA library of mouse macrophages by screening with a IL3 probe at low stringency. Its total lenght is 1262 bp with repeated sequence of 490 bp whose consensus sequence is "GAGAGGCA" and containing an O.R.F. (open reading frame). We find that both helixes can be transcribed into RNAs as shown by RNA hybridization. Computer analysis comparing all the repeated sequences described so far in the litterature has shown that the repeated we found had no homology with others. Total RNA from several differentiated and undifferentiated mouse cell lines was hybridized to a fragment containing only the repeated sequence. Results showed that there was a quantitative different expression reflecting the degree and the kind of differentiation in the cell lines tested. The sequence described here is also conserved through the evolutionary scale since we find it by cross-hybridization to DNA in yeast, sea urchin, Drosophila, Xenopus, chicken and human genome.

We conclude that the repeated sequence we characterized is a member of a new family probably coding for a protein related to differentiation and evolutionary widespread.

94 PROSOME, A NOVEL CLASS OF RNP PARTICLE ASSOCIATED WITH CYTOSKELETON AND NUCLEAR MATRIX.
Maria-Fatima Grossi de Sa (1), Francis Harper (2), Cezar Martins de Sa (1), Jayanta K. Pal (1), Omar Akhayat (1) and Klaus Scherrer (1). (1) Institut JACQUES MONOD, Université PARIS VII, 2 Place Jussieu, 75251 Paris Cedex 05 (France), (2) Institut de Recherches Scientifiques sur le Cancer, BP n° 8, 94800 Villejuif (France)

The prosome, a ScRNP particle was recently isolated, from the cytoplasm of different eukaryotic cells, as a subcomplex of the translationally repressed mRNP particles. The protein moeities of the prosome seem to be highly conserved among several biological systems examined, whereas the small RNA constitutents differ with respect to both the system and the type of mRNP fraction used to isolate the particle. A complex set of prosomal RNAs are present in preparations of heterogeneous mRNPs of duck erythroblasts, but the prosome from the purified globin mRNP of the same system contains only two species of small RNA. In order to assess the subcellular localization of prosomes, we have investigated the intracellular distribution of this particle in HeLa cells by both biochemical fractionation and in situ localization by immuno-fluorescence and immuno-electron microscopy using monoclonal antibodies raised against duck prosome proteins. Immuno-fluorescence and immuno-electron microscopy studies revealed that the prosome is associated with the characteristic fibers of the intermediate filaments, particularly with the cytokeratin network. This observation was further supported by immunoblots followed by subcellular fractionations where the antibody against the 27K prosome protein recognises the corresponding (27K) antigen in the cytoskeletal fraction, and a higher MW antigen (62K) in the nuclear matrix fraction. This results suggest that the repressed free mRNPs are associated to the cellular matrix possibly through the prosomes.

95 ISOLATION AND CHARACTERIZATION OF A DNA UPTAKE STIMULATING PROTEIN FROM THE CULTURE MEDIUM OF NEUROSPORA CRASSA

Gy. Tóth, I. Békési, M. Schablik, G. Szabó. Institute of Biology, University Medical School of Debrecen, H-4012 Debrecen, P.O.B. 1.

In the culture medium of the wall-less /slime/ strain of Neurospora crassa /FGSC 1118/ a protein appears at the early stationary growth phase, which stimulates the uptake of DNA into Neurospora slime cells, designated as DNA uptake--stimulating factor /DUSF/, [FEMS Microbiol. Lett./1981/ 10: 395-397]
The quantity of DUSF is measured by the amount of tritium-labeled-DNA uptake by Neurospora cells at standard conditions. The proteins were removed from the culture medium by ultrafiltration. Thereafter the proteins were chromatographed on DE-Cellulose 32 resin, followed by molecule sieving on Sephacryl-S300 column. The active DUSF was separated to homogeneity by preparative polyacrylamide gel electrophoresis. The molecular mass of DUSF estimated by molecular sieving, was 235 KDa. It has an isoelectric point of pH 5,4. In SDS gel electrophoresis, the molecule falls apart into subunits. The chemical composition of DUSF will be presented. The biological significans of DUSF will be discussed.

96 CELL-TO-CELL CONTACTS AND FUSION OF CELLS OF GRAMNEGATIVE BACTERIA INDUCED BY DIVALENT CATIONS.
V.L.Borovyagin (1), A.G.Sabelnikov (2), Yu.S.Tarahovsky (1), B.N.Ilyashenko (2). (1) Institute of Biological Physics, USSR Academy of Sciences, Puchshino, (2) Gamaleya Institute of Epidemiology and Microbiology, Moscow, USSR

Heavy aggregation of cells of gramnegative bacteria Escherichia coli and Pseudomonas putida in the presence of divalent cations (calcium, barium and magnesium) has deen detected by the light scattering technique. The level of aggregation increased sharply with the increase of cation concentrations between 40-400 mM and then remained constant. The process was not completely reversible since the cells washed extensively from the cations still exhibited residual and significant light scattering compared to untreated samples. Freeze-fracture and ultrathin sections electron microscopy of cells under these conditions revealed frequent and extensive formation of multiple cell-to-cell contacts and infrequent (10^{-2}-10^{-3}) fusion of cells. Tube-like membrane structures were frequently observed in the sites of contact. The evidence obtained suggest that irreversible aggregation and subsequent fusion of cells is a complex multistep polymorphic lipid transition process in which lipid polymorphic structures may be involved. Biological releavance of the results obtained are discussed in terms of genetic transfer during cell-to-cell contacts and fusion of bacterial cells observed recently in various bacteria.

97 LIPID POLYMORPHISM AND THE CATION INDUCED COMPETENCE OF GRAMNEGATIVE BACTERIA. A.G.Sabelnikov (1), I.A.Vasilenko (2), Yu.S.Tarakhovsky (3), V.L.Borovyagin (3). (1) Gamaleya Institute of Epidemiology and Microbiology, Moscow, (2) Lomonosov Institute of Fine Chemical Technology, Moscow, (3) Institute of Biological Physics, USSR Academy of Sciences, Pushchino, USSR.

Under the conditions of cation-dependent competence induction (the ability of DNA uptake) in gramnegative bacteria (E. coli, Ps. putida, etc.) ^{31}P-NMR spectra (recorded at 42 °C) of cell suspensions revealed the formation of a new and characteristic ^{31}P-NMR signal (BBRC 127, 464-472,1985). Under these conditions tubular and fingerprint-like lipid structures are observed on fracture faces. In addition, the cell surface reveals blabs and vesicles of various sizes which may be accounted for by the enhancement of isotropic signal in NMR spectra. Vesicles, blabs, and lipid aggregates in tetra- and hexagonal H_{II}-phases are often seen in ultrathin sections. The latter also reveal singular cylinder-like structures presumably composed of segregated LPS and partly of phospholipids. As evidenced by freeze-fracture experiments the formation of tubular and fingerprint-like lipid structures takes place in particle-free smooth areas of membrane surface. Tube-like structures and lipid aggregates both on fracture faces and on ultrathin sections have three distinct locations: at the outer side of the outer membrane, at the inner side of the plasma membrane, and within the cell envelope. It is suggested that polymorphic phase behavior of membrane lipids plays primary role in DNA uptake by the bacterial cells during cation-dependent genetic transformation and transfection. It may also play an important role in other processes of genetic exchange in nature.

98 REVERSE TRANSCRIPTION INTERMEDIATES OF MOBILE GENETIC ELEMENTS IN DROSOPHILA MELANOGASTER CELLS: DNA-RNA COMPLEXES AND VIRUS-LIKE PARTICLES.
T.V. Gorelova, M.Sh. Kubaneishvili, B.V. Semin, N.G. Schuppe. N.I. Vavilov Institute of General Genetics, USSR Academy

Hybrid molecules consisting of mobile genetic elements DNA and corresponding poly $(A)^+$ RNA were isolated from Drosophila melanogaster cultured cells. The presence of these complexes and their properties are consistent with the model of reverse transcription pathway of transposition and amplification of Drosophila mobile elements. This notion is strengthened by the existence in cultured cells of virus-like praticles containing sequences of mobile genetic elements (both RNA and DNA) and possessing reverse transcriptase-like activity. Particles with the same properties can also be isolated from the media during the cell culturing. Intracellular localization of DNA--RNA complexes and virus-like particles have been studied, and some of their physico--chemical properties have been determined. The data obtained permit us to present and discuss methabolic pathways of mobile genetic elements in Drosophila cells.

99 RNA OF TETRAHYMENA DURING DAPI-ARRESTED REGENERATION OF CILIA (II).

Wanda Krawczyńska(1), Barbara Kłudkiewicz(2). (1)M.Nencki Institute of Experimental Biology, Department of Cell Biology, 3 Pasteur Str.Warszawa, and (2)Institute of Biochemistry and Biophysic, Department of Comparative Biochemistry,36 Rakowiecka Str. Warszawa, Poland.

Deciliation of starved Tetrahymena cells induces synthesis of tubulin due to rapid activation of tubulin m-RNAs /Marcaud and Hayes, 1979; Eur.J.Biochem. 98,267/. When Tetrahymena cells were starved in the presence of a DNA-ligand 4',6-diamidine-2--phenylindole (DAPI), the recovery of cell motility was delayed /Krawczyńska, 1983; Acta Protozool. 22,33/. RNA extracted from DAPI-treated cells was separated on 2.4% polyacrylamide gel. In comparison with the control it was enriched in high molecular weight RNA /2nd Conference on Cell Biology, Warszawa, 1985/.

Analysis of the RNA samples fractionated on oligo(dT)-cellulose revealed that the content of poly(A^+)-RNA is lower in DAPI-treated cells than in untreated ones /1.43% and 1.89%, respectively/. However, the molecular weight of the poly(A^+)-RNA obtained from the DAPI-treated cells and the controls seems to be the same, as shown by electrophoresis on strongly denaturing gel.

These results suggest that DAPI disturbes processing of Hn-RNA, presumably by the inhibition of the poly(A)-tail formation.

100 THE INFLUENCE OF THE IMMUNOSUPPRESSIVE SESQUITERPENE OVALICIN ON THE METABOLISM OF RIBOSOMAL RNA. Hannes Diersch and Guido R. Hartmann, Institut für Biochemie der Ludwig-Maximilians-Universität, Karlstraße 23, D-8000 München 2, Federal Republic of Germany.

The proliferation of mouse lymphoma cells (strain S 49.1) is distinctly inhibited by 0.2 µM of the immunosuppressive sesquiterpene ovalicin (1) after an incubation for two cell cycles. Earlier observations pointed to the metabolism of ribosomal RNA as possible target (2). To analyse this effect the lymphoma cells were incubated for 90 min with (methyl-^{3}H)methionine. Subsequently the radioactively labelled methylated ribosomal RNA in the nuclei and in the cytoplasm was separated by gel electrophoresis. In cells which were exposed for 18 h to ovalicin much more 28S ribosomal RNA was found in the nuclei than in control cells. In the cytoplasm just the opposite was observed. These findings may be explained by the hypothesis that ovalicin inhibits the transport of 28S ribosomal RNA into the cytoplasm.

These investigations have been supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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101 CHARACTERIZATION OF DEVELOPMENTALLY AND ENVIRONMENTALLY REGULATED GENES IN POTATO

L. Willmitzer, Max-Planck-Institut für Züchtungsforschung, Abtlg. Schell; 5000 Köln 30, FRG

The differentiated state of higher plant cells is characterized by the differential expression of specific gene pools which respond to changes in either development or environment.
We have started a program with the aim to isolated and characterize differentially expressed genes from higher plants. We more specifically wanted to study genes expressed in a developmentally controlled (organ-specific) manner as well as genes induced by changes in the environment (more specifically wounding).
Potato was chosen for these studies as potato combines both the attractiveness of being an important crop plant which however is still reasonable well adapted to tissue culture techniques and transformation.
Results will be described upon the physical characterization of two developmentally controlled genes from potato, i.e. on the one hand a tuber-specifically expressed gene encoding patatin , the major storage protein of potato tuber and on the other hand a leaf-stem-specifically expressed, light-inducible gene of as yet unknown function. In addition a third gene encoding proteinase inhibitor II of potato has been isolated. This gene is under a rather complex control of both developmental (being tuber-specific) as well as environmental (being wound-induced in leaves) factors.
In addition to the physical analysis of these genes results will be presented concerning the regulated expression of chimaeric derivatives of these genes after introduction into both tobacco and potato plants.

102 BIOSYNTHESIS OF δ-AMINOLEVULINATE AND ITS REGULATION BY PLASTID AND NUCLEAR GENES:

C.G.Kannangara[1], S. Berry-Lowe[1], P. Bruyant[1], S.P. Gough[1], A. Kahn[2] and A. Schön[3]. 1. Department of Physiology, Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Copenhagen-Valby, Denmark. 2. Institute of Genetics, University of Copenhagen, Øster Farimagsgade 2A, DK-1353 Copenhagen K, Denmark. 3. Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut, U.S.A.

δ-Aminolevulinate is the major regulated intermediate in the chlorophyll biosynthetic pathway. The porphyrin ring of chlorophyll is formed by the condensation of 8 molecules of δ-aminolevulinate. We have studied the biosynthesis of δ-aminolevulinate in greening barley and elucidated several aspects of its regulation. δ-Aminolevulinate is synthesised from glutamate in the stroma of developing chloroplasts. Glutamate is first bound to the 3'end of δ-ALA-RNA catalysed by an aminoacyl ligase in the presence of ATP and Mg^{2+}. Glutamyl-δ-ALA-RNA is then converted to glutamate 1-semialdehyde by a NADPH dependent dehydrogenase which has yet to be characterised. In the last step the enzyme glutamate 1-semialdehyde aminotransferase catalyses the transamination of glutamate 1-semialdehyde to δ-aminolevulinate. This transamination reaction shows no requirement for added amino donors.

The components involved in δ-aminolevulinate synthesis have been isolated and separated into three fractions. A plastid stroma fraction was first passed through a Sephacryl S-300 column to remove high and low molecular weight contaminants and then sequentially through Blue-Sepharose, Matrix-gel red A and chlorophyllin (or heme-Sepharose) columns. The Blue-Sepharose binds the amino-acyl ligase and the dehydrogenase while the chlorophyllin (or heme-Sepharose) binds δ-ALA-RNA. Glutamate 1-semialdehyde aminotransferase does not bind to these affinity columns. Three of the components, δ-ALA-RNA, the aminoacyl ligase and the aminotransferase have been purified and characterised.

The primary structure of the δ-ALA-RNA fits the clover leaf model of a tRNA. Furthermore it contains several modified nucleosides and has a CCA sequence at the 3' end which can be removed using snake venom phosphodiesterase and replaced using nucleotidyl transferase. Removal of the CCA leads to loss of its glutamate acceptor activity and consequently the ability to reconstitute δ-aminolevulinate synthesis when combined with the other components. These activities are restored after the nucleotidyl transferase treatment. In the stroma of greening barley chloroplasts there are two other glutamate acceptor tRNAs which do not take part in δ-aminolevulinate synthesis. δ-ALA-RNA hybridizes to a Hind III/Pst I chloroplast DNA fragment but not nuclear DNA. Thus the gene for δ-ALA -RNA is located in the plastid DNA.

Glutamate 1-semialdehyde aminotransferase was one of the prominently labelled proteins when ^{35}S-methionine was incorporated into greening seedling leaves of barley. Labelled methionine is not incorporated into glutamate 1-semialdehyde aminotransferase in experiments using plastids isolated from greening barley leaves. Most likely, the aminotransferase is encoded by the nucleus, synthesized in the cytoplasm and transported into the chloroplast. We have been able to identify only one glutamate specific aminoacyl tRNA synthetase in the stroma of developing chloroplasts. δ-ALA-RNA and several $tRNA^{Glu}$ molecules are substrates for this enzyme. The dehydrogenase involved in δ-aminolevulinate synthesis in barley shows a stringent substrate specificity for glutamyl-δ-ALA-RNA since glutamyl-tRNAs are inactive. Possibly one glutamyl tRNA synthetase provides activated glutamate for both chlorophyll and protein synthesis.

The stroma fraction isolated from barley etioplasts show a significant amount of δ-aminolevulinate synthesizing activity. This activity (per seedling or per mg protein) increases 3 to 5 fold during the first 12 hours of light in seedlings which have been grown in darkness for 6 days. It is assumed that δ-ALA-RNA and the enzymes involved in the conversion of glutamate to δ-aminolevulinate are present in small amounts in the leaves of barley seedlings grown in darkness and increase 3 to 5 fold

during illumination. A brief illumination is sufficient to induce the increased formation of the components of the δ-aminolevulinate pathway although it is only in continuous light that δ-aminolevulinate synthesis is sustained, conceivably by the supply of higher levels of the cofactors NADPH, ATP and Mg^{2+}. In continuous darkness δ-aminolevulinate formation is reduced to a minimum in barley. Biochemical and genetic evidence indicate that this is achieved by a strong feedback inhibition of the dehydrogenase by heme.

Gabaculine is a potent inhibitor of the aminotransferase. Glutamate 1-semialdehyde accumulates in plants when chlorophyll synthesis is arrested by gabaculine treatment. Glutamate 1-semialdehyde overproducing mutants have been obtained in Chlamydomonas by screening mutagenised cells for gabaculine resistance. It is clear that the biosynthesis of δ-aminolevulinate in greening plants is a complex and unusual pathway, regulated by intermediates of the chlorophyll pathway. There is also a great deal of evidence, both from higher plants and algae, that the nuclear genome is responsible for the synthesis and regulation of chlorophyll. With the recent discovery of an RNA coded by the plastid genome and directly involved in δ-aminolevulinate synthesis, it is considered that regulation of the chlorophyll pathway results from a concerted interaction of nuclear and chloroplast genes.

103 TOTIPOTENT SOMATIC PLANT CELLS IN STUDIES ON REGULATION OF DEVELOPMENT.
L. Bögre, Maria Deak, Z. Olah, Tünde Praznovszky, J. Györgyey, Katalin Kalman, D.Dudits
Institute of Genetics, Biological Research Center, H.A.S. 6701 Szeged P.O. Box 521 Hungary

Cell differentiation in higher plants differs significantly from other eucaryotic organisms in the unique capability for restarting the developmental program. Under in vitro conditions the differentiated plant cells can be induced to divide and to form totipotent somatic embryos. In alfalfa cells 2,4-dichlorophenoxy acetic acid (2,4-D) is an essential factor in induction of totipotent stage. The embryogenic response is strongly determined by the genotype with few dominant genes. Using alfalfa cells we will present the subsequent events in embryo formation from leaf protoplasts with special emphasis on tubulin organization in dividing cells. The changes in gene expression pattern induced by 2,4-D will be monitored at mRNA level using a 2,4-D responsive cDNA probe, p4. This cDNA represents a gene coding for a cell wall protein (Jong Chan Hong et al. unpublished). In cultured alfalfa tissues the synthesis of mRNA homologous with p4 shows a correlation with the formation of differentiating embryogenic cells. Comparison between cells under inductive or non-inductive conditions will be extended to the protein level. The hormone action in reprograming differentiation pattern can be mediated by induction of kinases altering phosphorilation of characteristic proteins. The comprehensive analysis of somatic plant cells with competence to carry out a complete developmental program, upto formation of whole plant will outline the basic molecular processes in this unusual biological event.

104 A GENETIC APPROACH TO PLANT HORMONES.
Patrick J.King, Anne D. Blonstein, Jürg Oetiker, Martin Suter and Ludwig Wälder. Friedrich Miescher-Institut, Postfach 2543, 4002 Basel, Switzerland.

Despite the great amount of biochemical and physiological data gathered over many years on hormone metabolism and hormone effects on plants, most of the key questions are still open: in many cases the actual in vivo biosynthetic pathways are unclear, regulation of synthesis is not understood, cellular sites of synthesis are unknown, physiologically active receptors have not been identified. The reasons for these differences are manifold.
The induction and characterisation of mutants involved in hormone biosynthesis and hormone action is the most direct way to define the real in vivo situation. Our understanding of the gibberellins and of abscisic acid have benefited already from a genetic approach. More recent work in our laboratory, and in others, has led to the isolation of auxin mutants, showing resistance to auxin analogs or an induced auxin requirement. These mutants are being characterised genetically and biochemically.

105 SOMATIC HYBRIDISATION OF HIGHER PLANTS. By Yu.Gleba,V.A.Sidorov, and I.K.Komarnitsky. Institute of Botany, Ukrainian Academy of Sciences, Kiev, USSR.

Hybridisation of closely related species: a transmission genetics. In order to analyze the gene behavior under non-selective conditions, a series of special experiments have been performed. As a method for hybrid screening, visual identification of heteroplasmic fusion products followed by mechanical isolation and cloning has been utilized. As initial material, plant forms of Nicotiana were used that differed for various traits encoded for plasmagenes as well as nuclear genes. Progeny of 67 clones representing 11 species combinations were analyzed. The results obtained demonstrate that: 1) in the process of somatic hybridisation, nuclear genes are inherited both bi- and uniparentally; uniparental inheritance of nuclear genes is evidently connected with genomic rather than chromosome segregation; 2) cytoplasmic genes in the same process are inherited biparentally; heterozygosity for plastome genes is a relatively durable state; 3) mitotic segregation of plasmagenes in the process of hybrid multiplication is not independent; there are two separate linkage (cosegregation) groups of plasmagenes, one (plastome) including restriction sets of chloroplast DNA, plastome chlorophyll deficiency, polypeptide composition of RUBISCO large subunit, and tentoxin resistance, and another (chondriom) including restriction sets of mitochondrial DNA as well as cytoplasmic male sterility;4) at the interspecific level, there is no evidence for selective pressure against specific organelle types in the fusion products; 5) the restriction sets of mitochondrial DNA from most hybrids are not identical to parental sets suggesting recombination events; 6) plastome encoded characters show strict cosegregation, thus, plastome recombination is a rare event and can be ignored unless special selective pressure in favour of recombinants is applied.

Screening for plastome recombinants. We have fused protoplasts of the tobacco mutant carrying plastome-encoded characters of a) chlorophyll defficiency and b) streptomycin resistance with those of tobacco cms-analogs possessing cytoplasms of 5 different Nicotiana species. Green streptomycin-resistant phenotypes were recovered in combinations of the cytoplasms tabacum+undulata as well as tabacum+bigelovii. Analyses of restriction sets of chloroplast DNA as well as of several plastome-encoded characters demonstrated that plants in question possess recombinant types of chloroplast DNA.

Selection for asymmetric hybrids: fusion of somatic normal and irradiated cells.In first series of these experiments, mesophyll protoplasts of nitrate reductase-deficient mutants of Nicotiana plumbaginifolia were fused with irradiated (10, 30, 50, 100, and 200 Krds) leaf protoplasts of 9 species. Wild type cell colonies were found (irrespective of the dose of irradiation) in species combinations N.plumbaginifolia+N.sylvestris, N.p.+Atropa belladonna, N.p.+Beta vulgaris, and N.p.+Daucus carota.Biochemical and cytogenetic studies of cell lines and regenerants demonstrate that hybrids recovered possess different and reduced amounts of genetic material from irradiated "donor". In another series of experiments, Nicotiana plumbaginifolia plants that are resistant to kanamycin due to genetic transformation were used as a "donor". Isolated "donor"protoplasts were fused with mesophyll cells of 5 different species. Transfer of antibiotic resistance was demonstrated in all cases, and hybrids recovered turned out to be asymmetric.

106 CHLOROPLAST SEGREGATION AND RECOMBINATION AFTER PROTOPLAST FUSION AND SEXUAL CROSSES. P.Medgyesy, N.D.Thanh, R.Malone and A.Páy, Institute of Plant Physiology, Biological Research Center, P.O.Box 521, Szeged, H-6701 Hungary

Chloroplast transfer by sexual crosses is hampered by the uniparental-maternal inheritance of cytoplasmic organelles in most plant species. Transmission of paternal plastids, however, was observed in Nicotiana, using plants carrying streptomycin resistant plastids as pollendonors. Cell lines with paternal plastids in the offspring were selected as resistant calli induced from seedlings on streptomycin containing media. In the case of crossable species this sexual cybridization method offers a simple way to transfer chloroplasts solely (without mitochondria).

Protoplast fusion techniques further extend the possibilities of production of cybrid plants but a taxonomical limit is expected. Protoplast fusion experiments between distant species in Solanaceae suggest that plants with a new nucleus-chloroplast combination can be produced between Nicotiana tabacum and Salpiglossis sinuata (in an other tribus) but not between N.tabacum and Solanum species (in an other subfamilia).

Recently genetic recombination was described between chloroplasts of Nicotiana tabacum and N.plumbaginifolia after protoplast fusion. The parental lines differed in four genetic markers: streptomycin, lincomycin and tentoxin resistance / sensitivity and albinism /greening. Direct selection for a new combination of plastid markers seemed to be the key to recovering a clone with recombinant chloroplasts. Chloroplast recombination suggests a possibility for limited gene transfer in combinations where the transfer of whole chloroplasts is prevented by an incompability at organic or cellular level.

107 DIRECT GENE TRANSFER TO PLANTS AND INHERITANCE OF FOREIGN GENES IN TRANSGENIC PLANTS.
Ingo Potrykus, Jerzy Paszkowski, Michael Saul, Ioan Negrutiu, Raymond D. Shillito. Friedrich Miescher Institut, P.O.Box 2543, CH-4002 Basel, Switzerland.

Incubation of protoplasts with a foreign gene under the control of plant expression signals leads to stable integration into the host genome. The gene is expressed in plants regenerated from protoplast-derived clones and it is inherited in successive sexual generations according to Mendelian laws. In the majority of the transgenic plants analysed so far, the foreign gene is present a a single dominant factor. However, plants having the gene integrated at two, three and multiple independant loci, as well as plants showing reciprocal differences in the transmission of the gene, have also been identified. Proof for transformation is based on strict correlation between treatment, phenctype, inheritance, physical presence, and activity of the foreign gene. Theforeign gene is found to be stably maintained over, so far, six sexual generations and without any selective pressure. However, there are also clones which are characterized by various degrees of instability of the gene. A partially optimized protocol which includes electroporation, heatshock and polyethyleneglycol, yields routinely rates of stable integrative transformation of functional copies of foreign genes of 1-3% with Nicotiana tabacum and of 1-0.1% with other species such as Petunia hybrida, Hyoscyamus muticus, Nicotiana plumbaginifolia, etc. Hostrange limitatins were not found so far and foreign genes have been transferred also to graminaceous monocots (cereals) which are, so far, not accessible to Agrobacterium-meciated gene transfer. Gene transfer to cereal plants has, however, not been achieved yet because plant regeneration from cereal protoplasts is still a problem. Treatment of protoplasts with total genomic DNA isolated from plants carrying one copy of a selectable marker gene per haploid genome led to the recovery of transgenic plants with this gene. Co-incubation of non-selectable genes and a selectable marker gene produced up to 88% cotransformation. The localisation of the foreign gene in transgenic plants was made visible by in-situ hybridisation to metaphase chromosomes of a radioactively labelled prcbe.

108 GENETIC TRANSFORMATION OF HIGHER PLANT CELLS USING MICROINJECTION. By P.V.Melnikov, T.P.Pasternak, V.M.Andrianov, E.S.Piruzian, K.M.Sytnik, and Yu. Gleba. Institute of Botany of the Ukrainian Academy of Sciences, Kiev; Institute of Molecular Genetics, Academy of Sciences, Moscow, USSR.

Fate of DNA introduced into plant cells using microinjection of plasmids has been studied. Regenerating (3-5 days of culture) mesophyll or callus protoplasts of both Nicotiana tabacum and N.debneyi were used as recipient. Plasmids used included constructions of PBR 322 carrying specific sequences of T-region (onc-genes or gene for nopaline synthase), as well as PBR 322 -derivatives carrying structural part of gene for neomycin phosphotransferase under different promoters (nos of Ti-plasmid,gene VI of CaMV, and early region promoter of SV 40). Injected cells were transferred into droplets of nutrient medium and cultured under non-selective conditions for 2-4 weeks. Subsequent transfer onto selective media revealed transformants in most experiments, the transformation frequency being up to 40 % out of total amount of recovered colonies. Analyses using blot-hybridization has confirmed the presence of the donor DNA sequences in selected cell lines. Potential of microinjection as a transformation method will be discussed.

109 SPATIAL SEPARATION OF PARENTAL GENOMES IN HYBRIDS OF SOMATIC PLANT CELLS. By A.S.Parokonny, V.P.Momot, V.N.Kotov, and Yu.Gleba. Institute of Botany, Ukrainian Academy of Sciences, Kiev, USSR.

Spatial arrangement of chromosomes in metaphase nuclei of somatic cell hybrids Nicotiana chinensis+ +Atropa belladonna, N.plumbaginifolia+A.belladonna, and N.plumbaginifolia+N.sylvestris has been analyzed. It has been found that during first division, chromosomes of two parents are not randomly intermixed in metaphase plate, rather they are grouped in segments, each containing predominantly chromosomes of one parent. Spatial separation of parental genomes is conserved during long-term (6-48 months) culturing of hybrid cells, although topology of separation is changed from "segment" to "radial" (chromosomes of one species being in the center and those of another occupying peryphery of the metaphase plate). Analyses of cells grown for several days in the presence of colchicine (100, 330, and 1000 mg/l) and afterwards cultured for 1-7 months in colchicine-free medium show that colchicine induces disorders in chromosome spatial arrangement, and that these alterations are heritable. Therefore, the phenomena studied are probably of epigenetic nature. Possibilities of using somatic cell hybrids as a model for analysing the mechanisms of mitosis are discussed.

110 SOMACLONAL VARIATION INDUCED IN CALLUS CULTURES OF RICE

L.E. Heszky(1), Li Su Nam(1), A.H.Nagy(2), E.Kiss(1), Zs.Horváth(3), I.Simon-Kiss (4), M.Hajós-Novák(1), (1) Dept.Plant Breeding, Agricultural University Gödöllő, Hungary, (2) Dept. Genetics Eötvös L. University, Budapest, Hungary, (3) Research Centre for Agrobotany, Tápiószele, Hungary, (4) Institute of Irrigation, Szarvas, Hungary

The objective was to identify variants of agronomic importance of rice somaclones. Somaclones of four commercial rice cultivars were regenerated from cultured cells in vitro. Flowering date of plants, amino acid content of seeds and chromosomal variations in mitoses were tested. The variants were analyzed by electrophoresis, too. Comparisons have been carried out between somaclones of different origin e.g. anther callus immature inflorescence callus of haploids and plumule meristem callus. The possible application of somaclones for increasing the genetic variability of breeding materials of rice is discussed.

111 SIGNAL TRANSDUCTION BY GROWTH FACTORS

W. Moolenaar, L. Defize, B. Tilly, W. Kruijer, A. Bierman, S. de Laat
Hubrecht Laboratory, Uppsalalaan 8, 3584 CT Utrecht, the Netherlands

The proliferation of cells *in vivo* and in culture is regulated by polypeptide growth factors, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). Growth factors initiate their action by binding to specific cell surface receptors. Receptor occupancy triggers a cascade of physiological changes in the target cell which ultimately lead to DNA synthesis and cell division. Immediate consequences of receptor activation include tyrosine-specific protein phosphorylation, inositol lipid breakdown, a sustained increase in cytoplasmic pH (pH_i) and a transient rise in free Ca^{2+}. The rise in pH_i has a permissive effect on DNA synthesis and is mediated by an otherwise quiescent Na^+/H^+ exchange mechanism in the plasma membrane, which is turned on by protein kinase C through the formation of diacylglycerol. The rapid Ca^{2+} signal is due to either release from internal stores (PDGF) or net entry via a voltage-independent pathway in the plasma membrane (EGF). Phorbol esters, acting via kinase C, inhibit the growth factor-induced Ca^{2+} signals without affecting resting Ca^{2+} levels. Artificially raising free Ca^{2+} mimics growth factors in inducing the expression of the c-*fos* and c-*myc* proto-oncogenes.

Monoclonal antibodies against the human EGF receptor can act as partial agonists in that they activate the tyrosine-specific protein kinase without inducing inositol lipid breakdown or any of the ionic signals. These antibodies fail to induce DNA synthesis when added to quiescent fibroblasts, indicating that inositol lipid breakdown and the Ca^{2+} and pH_i signals can be dissociated from tyrosine--kinase activity and suggesting that these signals are indispensable for the stimulation of cell proliferation.

112 REGULATION OF EPIDERMAL CELL PROLIFERATION BY EPIDERMAL GROWTH FACTOR (EGF) AND RELATED PEPTIDES.

G.P.M. Moore, P. Wynn, P. Djura and P. Van Dooren, CSIRO, Division of Animal Production, Prospect, NSW, 2148, Australia

The skin of mammals contains discrete epidermal or epidermally-derived cell populations which differ markedly in their patterns of proliferation. For example, the mitotic index (MI) of the epidermis of sheep lies between 0.1 - 0.5% with a probable cycle interval of 100 h, whereas the MI of wool follicle cells of epidermal origin ranges from 2.6 - 5.7% with a cycle time of 21 h. These patterns are reversed by mouse EGF or EGF produced by recombinant DNA methods (Coopers Animal Health, Australia). A stimulation of the MI of basal epidermal cells was observed in skin of sheep treated with EGF, whereas the MI of follicle bulb cells was reduced to near zero. The mechanisms by which EGF effect these changes are not clear, although the increase in numbers of proliferating cells in the epidermis may have been due to an increase in the rate at which the cell population progressed through the cycle and by the recruitment of quiescent cells to the cycling compartment.

Specific, high affinity receptors mediating these cellular responses were detected by autoradiography of frozen skin sections incubated with ^{125}I-EGF and by immunocytochemistry using antiserum to EGF receptors (provided by J. Schlessinger). EGF receptors were concentrated in the basal cells of the epidermis and the bulb and outer root sheath cells of the follicle. Together, these observations indicate that an endogenous EGF-like molecule regulates epidermal cell proliferation. In an endeavour to isolate this material, we have located an EGF-like protein in the sheep brain. Extracts were chromatographed on a G 100 Sephadex column and the EGF-containing fraction identified with an antiserum to mouse EGF in immunodiffusion discs. The partially purified sheep EGF displaced ^{125}I-EGF in a radioreceptor assay using ovine skin membrane particles and effected precocious eyelid opening in neonatal mice. Further purification of the sheep EGF was obtained by reverse phase HPLC using an acetonitrile/water gradient. Retention time and absorbance profile of the active fraction were similar to mouse EGF.

113 AVAILABILITY OF PDGF RECEPTOR, AS AFFECTED BY THE GROWTH CONDITIONS

R.Zippel°,E. Sturani°, P.M. Comoglio°°, L. Toschi°, L. Alberghina°, (°)Dipartimento Fisiologia e Biochimica Generali,V. Celoria 26, Milano,(°°)Istituto Istologia,C.so M. D'Azeglio 52,Torino

The PDGF receptor is endowed with tyrosine kinase activity and becomes rapidly phosphorylated in tyrosine following exposure of intact quiescent Swiss 3T3 fibroblasts to the factor. Antibodies against phosphotyrosine (P-tyr) recognize the phosphorylated form of the receptor which thus can be detected as a very sharp 170 Kd band on Western blotting of SDS PAGE fractionated total cell protein (Zippel et al, Biochim. Biophys. Acta, 1986 in press). At high PDGF concentration the intensity of the band is maximal after 5 min and decreases thereafter, while the kinetics of band appearence is modified at low PDGF.

The PDGF receptors present on the cell surface can be detected by their ability to become phosphorylated in tyrosine following a short treatment of cells with high PDGF concentration. By this approach we showthat treatments with PDGF cause the disappearence of the functional receptors from the cell surface the higher the PDGF concentration, the faster being the decrement of the receptors. Moreover the receptors, detected as indicated, are present in low amount during exponential growth in the presence of serum and in larger amount when the culture is approaching the stationary phase. The scarcity of the PDGF receptors during exponential growth appears to be due to a continuous downregulation by the PDGF present in serum: in fact the shift of growing cells to platelet poor plasma or the addition of suramine (which reversibly inhibits the binding of PDGF to its receptor) allow the reappearence of the receptors on the cell surface. Moreover both treatments cause these cells to become arrested in G1 confirming that postmitotic cells require to interact with PDGF for continuous cycling (Sturani et al,Exp.CellRes.153,135).

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114 EPIDERMAL GROWTH FACTOR-STIMULATED PHOSPHOPROTEIN SECRETION BY CULTURED RAT KIDNEY FIBROBLASTS.

Bert Binas, Richard Grosse. Central Institute of Molecular Biology, Academy of Sciences of the GDR, 1115 Berlin, Robert-Rössle-Straße 10, German Democratic Republic

Confluent cultures of Normal Rat Kidney Fibroblasts, clone 49 F, release a 58 kDa phosphoprotein (pp58) into the medium as detected by SDS-PAGE and autoradiography after labelling the cells for three hours with ^{32}P-orthophosphate. pp58 is not a dominant intracellular phosphoprotein, but it is the major phosphoprotein in the medium. Incubation of the cells with EGF during the labelling period results in dramatic increase of secretion of pp58, while prolonged preincubation of the cells in low serum reduces it severalfold. The effect of EGF is reversible and can be prevented by actinomycin D. The secretion is suppressed by cycloheximide and ammonium chloride. A secretory protein with a slightly higher apparent molecular weight than 58 kDa can be labelled with ^{35}S-methionine and is also selectively enhanced by EGF. Several other growth factors, like PDGF, NGF, TGF-ß, and insulin do not affect the secretion of pp58. In contrast, the synthetic tumour promoter, TPA, mimics the action of EGF.

115 DIFFERENTIAL EXPRESSION OF THE SRC GENE TRANSCRIPTS DURING DEVELOPMENT OF D. MELANOGASTER

Orit Segev and Ze'ev Lev, Department of Biology,
Technion - Israel Institute of Technology, Haifa 32000, Israel.

The expression of the Drosophila src gene was studied. The gene, located at 64B, extends over at least 7 kb of genomic DNA, and codes for three transcripts which are 3.0, 4.4 and 4.8 kb long. Their 3'-ends were identified and tentatively located on the physical map of the gene. All three transcripts are in the same polarity and contain the information required for translating the protein kinase domain common to all members of the src gene family. The Drosophila src transcripts are expressed in two different patterns during development. The 3.0 and the 4.4 transcripts are abundant in unfertilized eggs and during embryogenesis. Their prevalence decreases under the limit of detection in the larval and pupal stages, and they appear again in adult flies. The larger 4.8 kb transcript is undetectable in unfertilized eggs. It starts accumulating between two and five hours after fertilization, and is expressed continuously during development. Other Drosophila oncogenes [including abl, ras1, ras2 and ras3 and epidermal growth factor receptor (erbB)] are also developmentally regulated (1-3), and the Drosophila src expression is tissue specific (4). These results suggest that cellular oncogenes, apparently serving as mitotic signals, may also have a role in developmental processes.

1) Telford et al. (1985) EMBO J. 4, 2609-2615.
2) Lev et al. (1985) Mol. Cell. Biol. 5, 1540-1542.
3) Lev et al. (1985) Dev. Biol. 110, 499-502.
4) Simon et al. (1985) Cell 42, 831-840.

116 THE ROLE OF PROTOONCOGENES IN CARCINOGENESIS PRESUMABLY PRODUCING INHIBITORS OF CELL PROLIFERATION.

A. Balázs., Á. Tapolcai, Gy.Heténvi, Zs. Szántó
Institute of Experimental Medicine, Hungarian Academy of Sciences, H-1450 Budapest, POB 67, Hungary

Protooncogenes (c-onc) are regarded as sequences coding the production of specific proteins analogue in their chemical structure and function that of growth factors (e.g. PDGF), their receptors (EGF) or cytoskeletal components (e.g. tubulin). Affected by retroviral or other oncogenes the first step of carcinogenesis consists of GF overproduction. Some experimental data, e.g. the somatomedin-somatostatin or the prolactin-PIH antagonism prove that cell proliferation is submitted to dual control of positive and negative growth factors. Consequently we presume the existence of protooncogen sequences coding the synthesis of proliferatory inhibitors, too. Defects of c-onc-s leading to decreased production of inhibitors may presumably regarded as casual agents of oncogenesis, too. The chemical purification and cell biological effects of a nucleopeptide inhibiting both normal and leukaemic myeloid proliferation selectively is demonstrated in details.

117 EFFECT OF v-src GENE EXPRESSION ON THE DENSITY-DEPENDENT INHIBITION OF GROWTH.

Christiane BLAT+, Jacqueline VILLAUDY°, Geneviève DESAUTY+, Alice GOLDE°, and Louise HAREL+.
+ I.R.S.C. BP.N°8 VILLEJUIF (France).°Institut Curie. 75005 PARIS (France).

Similarities between the mode of action of growth factors and the oncogene product (pp60 src protein) of Rous Sarcoma virus have been described. However, a major difference is that addition of growth factors does not induce a malignant transformation of cells. The present work proposes a hypothesis concerning this difference. Various data suggest that density-dependent inhibition (DDI) of growth in non-transformed cells is due to the diffusion of growth inhibitory molecules. Inhibitory factors of 45K (IDF45) and 12K have been fractionated. We assume that the stimulation of DNA synthesis induced by growth factor addition to dense quiescent cultures of non-transformed cells leads to an increase in the activity of autocrine inhibitory molecules in such a manner that the growth factor stimulatory effect is only transient, and cells re-enter the Go phase. On the contrary, the stimulation of DNA synthesis by v-src transformation would not be counterbalanced by inhibitory diffusing factors and cells would not enter Go phase. We present preliminary results which support this assumption. Dense quiescent cultures of chick embryo fibroblasts infected by Ny68 virus (ts mutant for transformation of Rous Sarcoma virus) were stimulated to proliferate either by addition of growth factors in cultures maintained at 41°C or by expression of transformation (by the cell transfer from 41° to 37°C, the permissive temperature for expression of transformation). Stimulation of DNA synthesis by growth factors was totally inhibited by the inhibitory diffusing factors of 45K (IDF45) whereas the stimulation of DNA synthesis produced by transformation was poorly decreased by IDF45.

118 COMMON MECHANISM FOR THE CONTROL OF GLYCOLYSIS BY HORMONES, ONCOGENES, AND TUMOR PROMOTERS IN UNDIFFERENTIATED CELLS. Guy G. Rousseau, Lisardo Bosca, Ann M. Loiseau, Marina Mojena and Louis Hue. International Institute of Cellular and Molecular Pathology, and Louvain University Medical School, Avenue Hippocrate 75, B-1200 Brussels, Belgium.

Many types of undifferentiated cells maintain a high glycolytic rate under aerobic conditions. A stimulation of glycolysis is also observed upon exposure of quiescent cells to hormones and tumor promoters, and upon transformation by certain oncogenes. The discovery in liver (Van Schaftingen, Hue and Hers, Biochem. J. 192: 897-901, 1980) of fructose 2,6-bisphosphate (Fru-2,6-P2), the most potent stimulator of phosphofructokinase (PFK-1), led us to investigate this ill-understood phenomenon. The rate, key enzymes, and metabolites of glycolysis were determined in rat hepatoma (HTC) cells exposed to glucocorticoids, and in chick embryo fibroblasts (CEF) exposed to insulin or phorbol 12-myristate 13-acetate (PMA), or transformed by the Rous (RSV) or Fujinami (FSV) sarcoma virus. Lactate release was increased in all cases. Fru-2,6-P2 was identified in HTC cells and in CEF, and was shown to stimulate their PFK-1. In HTC cells, dexamethasone increased the concentration of Fru-2,6-P2 and the Vmax of the enzyme that catalyzes its synthesis, phosphofructo-2-kinase (PFK-2). These changes also took place in CEF treated with insulin or PMA, or transformed by RSV or FSV. This was mimicked by PMA analogues known to stimulate or to bind to protein kinase C, suggesting a role of this kinase in the PMA effect on Fru-2,6-P2. The effect of PMA was not additive to that of oncogene expression. In contrast to the liver enzyme, the PFK-2 of HTC cells or CEF was not inhibited by cyclic AMP, suggesting that undifferentiated cells possess a peculiar PFK-2 isozyme. These data point to the Fru-2,6-P2/PFK-2 system as critical for the control of glycolysis by glucocorticoids, mitogens, phorbol esters, and class-I oncogenic proteins. We thank Dr. J. Ghysdael for providing the viruses. The help of M.A. Gueuning and L. Maisin is gratefully acknowledged. Work supported in part by the CGER and the FRSM (Belgium).

119 EARLY STIMULATION OF ATP TURNOVER BY EGF + INSULIN. RELATION TO DIFFERENT EARLY EVENTS. S. Talha and L. Harel. I.R.S.C. BP N°8. 94802 VILLEJUIF CEDEX.

We previously demonstrated a rapid increase in ATP turnover after addition of epidermal growth factor (EGF) and insulin to quiescent cultures of Swiss 3T3 cells. We showed that cytochalasin D specifically inhibited the stimulation by growth factors of ATP turnover without any inhibitory effect on unstimulated cells. The inhibitory effect of cytochalasin D was transient. We hypothesize that the rapid and transient change in cell movements induced by growth factors could be responsible for the early increase in ATP degradation and turnover. The relationship between this increase in ATP and the activation by growth factors of Na^+/H^+ and Na^+/K^+ exchange systems was studied.

Our results show that alkalinization of the medium enhances ATP turnover but they do not support the assumption that stimulation by growth factors of the Na^+/H^+ exchange induces an increase in ATP turnover since this increase was not inhibited by amiloride. Conversely, when ATP synthesis was abolished, the increase, in intracellular pH, by growth factors, was significantly decreased.

120 **THE INFLUENCE OF DANSYLCADAVERINE ON ENDOCYTOSIS OF EPIDERMAL GROWTH FACTOR AND INITIATION OF EARLY CELL REACTIONS AND DNA SYNTHESIS.** N.N.Nikolsky, N.V.Kudryavtseva, A.M.Nesterov, A.B.Sorokin. Institute of Cytology, Academy of Sciences of the USSR, Tikhoretskyi pr., 4, 194064, Leningrad, USSR.

Dansylcadaverine (DC) is often used in analysis of the role of receptor-mediated endocytosis. However the results are contradictory. In the present work the influence of DC on the DNA synthesis, uridine phosphorylation, phosphorylation of membrane proteins and binding, clustering, internalization and degradation of epidermal growth factor (EGF) was investigated. It was found that 0.1 mM DC inhibits such EGF-induced reactions as uridine phosphorylation, DNA synthesis but it does not influence the binding, clustering, internalization or degradation of EGF. The DC concentration of 0.5 mM inhibits EGF internalization. Time dependence of the DC blocking effect on uridine phosphorylation allows to conclude that DC affects primarily the "activated" form of uridine kinase. The blocking effect of DC is maintained in the presence of monensin that prevents the accumulation of DC in acidic cell compartments. DC exerts no influence on pH of lysosomes and endosomes. The evidence obtained show that the DC action does not result from pH-dependent accumulation of DC and therefore can be accounted for by insertion of DC molecules into membranes. It is suggested that the inhibitory effect of DC on the uridine phosphorylation and DNA synthesis is realized at the stage of EGF receptor processing in endosomes and/or Golgi apparatus.

121 IMMUNO-GOLD LABELING OF EGF-RECEPTORS IN CULTURED A431 CELLS.

J. Boonstra (1), N. van Belzen (1), P. van Maurik (2), F.A.C. Wiegant (1) and A.J. Verkley (1).
(1) Department of Molecular Cell Biology, University of Utrecht, Padualaan 8, 3584 CH Utrecht,
(2) Hubrecht Laboratory, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.

The recent development of a number of methods, i.e. cryo-ultramicrotomy, freeze-etching, label-fracture, surface replication and dry-cleavage, combined with immuno-gold labeling provides the possibility to study the distribution of cell-surface and intracellularly located antigens at the ultrastructural level.We have applied these methods in the visualization of EGF-receptors in A431 cells, using a monoclonal anti EGF-receptor antibody designated 2E9.

Cryo-ultramicrotomy is demonstrated as a useful method to visualize intracellularly located EGF-receptors. A dense labeling was observed on the plasma membrane and intracellularly on a network of tubular and vesicular membranes, multivesicular bodies, lysosomes and Golgi membranes, the method is therefore suitable for ultrastructural studies of EGF-receptor biosynthesis and receptor-mediated endocytosis.

An important feature of growth factor receptor interaction in the mitogenic response appears to be the redistribution of cell-surface located receptors, the so-called receptor clustering. Freeze-etching and label-fracture are both convenient methods to analyze the lateral distribution of cell-surface located EGF-receptors and therefore can be used to study the clustering phenomena in detail.

Finally evidence has been obtained of an association of growth factor receptors and cytoskeletal elements. We have used surface-replication and dry-cleavage methods on immuno-labeled cells, in the presence or absence of Triton X-100, to visualize the association between EGF-receptors and cytoskeletal elements on ultrastructural level.

122 SECOND MESSENGERS GENERATED BY NERVE GROWTH FACTOR IN PC12 CELLS. L.M.Vicentini, A.Pandiella, A.Malgaroli, T.Pozzan and J.Meldolesi. Department of Pharmacology,University of Milano, Milano, Inst. of Pathology, University of Padova, Padova, Italy.

Seven-10 day treatment with nerve growth factor (NGF) induces rat pheochromocytoma cells (PC12) to acquire a neuronal-like phenotype. In addition, during the first 24-36 hrs NGF has a mitogenic effect on PC12 cells. Although NGF effects have been studied for almost two decades, the intracellular signals generated by the interaction of the factor with its specific receptor have never been elucidated. By using the fluorescent probes quin2 and fura-2 we show for the first time that NGF induces a two fold increase in intracellular Ca^{++} concentration in both PC12 cell suspensions and single, attached cells. The NGF-stimulated Ca^{++} rise has a delay of about 30 sec, is abolished in Ca^{++}-free medium and it is not inhibited by the voltage dependent Ca^{++} channel blocker verapamil. The lack of effect of NGF on membrane potential is also confirmed by direct measurements with the fluorescent voltage probe bisoxonol.

We also show that NGF induces an intracellular alcalinization which is blocked by amiloride and abolished in a Na^{+}-free medium. This indicates that NGF stimulates Na^{+}/H^{+} exchange in PC12 cells. The possible effect of NGF on phosphoinositide metabolism is now being investigated. Since Ca^{++} and pH rises are signals typically stimulated by growth factors, it is possible that NGF-induced Ca^{++} and pH rise are also related to its mitogenic effect. Whether these early membrane events are necessary for the NGF-induced differentiation process remains to be established.

123 THE GROWTH STIMULATION EFFECT OF OPIOID PEPTIDES ON NERVE TISSUE IN CULTURE. O.B. Ilyinsky, M.V. Kozlova, V.U. Kalentchuk, E.S. Kondrikova, M.I. Titov, Z.D. Bespalova. All-Union Cardiology Research Centre, Academy of Medical Science, 15A, 3-d Cherepkovskaya, Moscow, USSR. 121552.

The effect of leu- and met-enkefalin β-γ-endorfins and 5 synthetic analogs of leu-enkefalin was studied on organotypic culture of rat sympathetic, spinal ganglia and spinal cord. All peptides were able to stimulate fiber outgrowth from explant, increase the number of glia and fibroblasttypic cells in the growth zone. The reaction was about 2 times more then the control value. The effect was demonstrated in a range 10-8 - 10-14 M. The surviving of neurons of the spinal ganglia is 2,4 times more than the control. Naloxon (10-5-10-7 M) doesn't inhibit the effect of peptides and stimulates the growth of cultures. The results showed that opioid peptides show significant growth stimulation effect on the tissues of peripheral and central nerve system in culture. Besides stimulation, the outgrowth of neurites and surviving of neurons they changed the migrational properties of glia and may increase it's proliferation. It is suggested that opioid peptides can be used as nonspecific growth factors for the nervous system.

124 EFFECT OF GROWTH HORMONE AND INSULIN-LIKE GROWTH FACTOR 1 ON DNA SYNTHESIS AND COLONY FORMATION BY CHONDROCYTES FRACTIONATED FROM RAT RIB GROWTH CARTILAGE.
Kjell Madsen, Ann-Marie Makower, Joanna Wroblewski, Ulf Friberg. Department of Histology, Karolinska Institutet, S-104 01 Stockholm, Sweden

According to the somatomedin hypothesis, the growth promoting effect of growth hormone (GH) is mediated by GH-dependent plasma peptides - somatomedins - that are produced mainly in the liver. Somatomedin A and C have recently been shown to be identical with insulin-like growth factor 1 (IGF-1). However, experimental data accumulated during the last few years suggest that GH has a direct effect on DNA-synthesis and matrix molecule production by chondrocytes in the epiphyseal growth plate. In view of these contradictory observations several models for the mode of action of GH may be proposed. For example, GH could induce the expression of somatomedin receptors on the chondrocyte plasma membrane. Another possibility is that GH may induce local synthesis of somatomedin-like factors. These two models do not necessarily exclude each other. In an attempt to find out more about the mechanism for growth hormone action, chondrocytes were isolated from rat rib growth cartilage and fractionated by centrifugation in a discontinuous Percoll gradient, and the fractions were subsequently cultured as monolayers or in semisolid agarose. The cultures were treated with Rat GH or biosynthetic IGF-1 and DNA synthesis, and (for agarose cultures) colony number, size, and morphology was determined. Both GH and IGF-1 stimulated the DNA synthesis of the cultured cells, but GH only stimulated DNA synthesis in small, high-density cells, whereas IGF-1 also stimulated larger cells, presumably from the hypertrophy zone, to proliferate. In the agarose cultures, GH selectively increased the number of large colonies, whereas IGF-1 caused an increase mainly in smaller colonies (<16 cells). Only the high-density fraction formed a significant number of colonies in agarose. The results support the idea of a direct effect of GH on chondrocytes. The target cells for GH appears to be small, high-density cells, presumably from the upper part of the growth plate.

125 IN VIVO MITOGENIC EFFECT OF CALCIFERIN AND CATHEPSIN ON LIVER AND THEIR UBIQUITOUS PRESENCE IN THE VERTEBRATES BLOODS. Hiroshi Terayama, The Kitasato Institute, 5-9-1 Shirokane, Minatoku, Tokyo-108, Japan.

Calciferin prepared from the bovine blood stimulates the release of cathepsin D from liver lysosomes or erythrocytes (or ghosts) in vitro, and also elevates cathepsin D in the blood in vivo. Both calciferin and cathepsin D in the blood were elevated concomittently soon after partial hepatectomy, and they exerted the mitogenic effect (DNA synthesis or mitosis) on the intact liver in vivo (but not on the primary hepatocyte cultures), suggesting that they may somehow be involved in liver regeneration.

Calciferin was only detected in the parathyroid except the blood. The blood calciferin was deleted after parathyroid-thyroidotectomy as well as pancreatectomy. In the latter case, glucagon (but not thyroxine or insulin) injections restored the blood calciferin. The secretion of calciferin from bovine parathyroid slices was dependent upon the physiological concentrations of glucagon.

Calciferin was detected in the sera from almost all vertebrates (except a kind of shark and bovine fetuses) including mammals, birds, reptiles, amphibians and fishes. The ultimibranchial body in the lower vertebrates may be the hormone (calciferin)-producing organ like the parathyroid in the higher vertebrates. The calciferin action on the erythryocytes or ghosts seems to be mediated by the mobilization of plasma membrane-bound Ca^{2+} ions and the activation of a Ca^{2+}-dependent protease. Calciferin in the human bloods appears to vary in the level under certain pathological conditions. Ref: Intl. J. Biochem. 16, 147-153 (1984), and 17, 949-955 (1985), Comp. Biochem. Physiol. 77A, 39-44 (1984), Exp. Cell Res. 151, 273-276 (1984).

126 INCREASED BINDING OF INTERFERON IN BLOOD PLASMA FROM TUMOUR-BEARING ANIMALS AS A POSSIBLE FACTOR IN THE BLOCKING OF ITS BIOACTIVITY IN MALIGNANT GROWTH.

G.V.Glinsky, A.B.Ivanova, V.B.Vinnitsky. Institute for Oncology Problems Acad.Sci. Ukr.SSR, 45, Vasilkovskaya street, 252022, Kiev-22, USSR

Peptide-protein binding in blood plasma from tumour bearers is altered for various neuropeptides, hormones and growth factors. But whether or not interferon(IFN)-binding proteins capable of modifying its bioactivity are present in blood plasma is unknown. We have investigated the binding of IFN-α in blood plasma from normal and tumour-bearing animals in two independent analytical systems: 1) quantitative analysis of the free form of IFN-α (RIA "Abbot") with the ultrafiltration of plasma samples containing IFN-α standard through YM-30 "Amicon" filters (nominal molecular weight cut-off 30.0 KD); 2) competing binding test with anti-IFN-α monoclonal antibodies (anti-IFN-α MA) as anti-IFN-α MA-coated beads and ^{125}I-anti-IFN-α MA. The binding of IFN-α is increased in blood plasma from tumour bearers bringing about 1.6-fold decrease in the level of IFN-α free form (percentage of IFN-α free form giving 21.0±1.70% and 13.0±0.31% in blood plasma from the control and tumour-bearing animals, respectively). Tumour-associated polyamine-containing peptides isolated from blood plasma of tumour bearers, but not the free spermidine, decrease in vitro the level of IFN-α free form by 1.3 times. Competing binding test with anti-IFN-α MA showed that the binding of IFN-α in blood plasma from tumour bearers is enhanced by 4.8 times (3.7±0.10 IU per mg protein and 17.8±0.55 IU/mg of bound IFN-α in plasma from the control and tumour-bearing animals, respectively). The analysis of Scatchard plot ascribes the differences in the binding to the increased number of high affinity IFN-α binding sites. The data obtained reveale high affinity binding of IFN-α in blood plasma, probably, to a carrier protein. Significant increase in the binding of IFN-α in blood plasma from tumour bearers may provide a mechanism which blocks the bioactivity of IFN as negative growth factor, antiviral and antiinflammatory agent in malignant growth.

127 CAPACITY FOR LOCAL INFILTRATION, AND METASTASIS BY BLOOD-BORNE AND LYMPHATIC ROUTES, IN CELLS BEARING TRANSCRIPTIONALLY ACTIVE HUMAN ONCOGENES. K.A. Rose (1), R.G. Morris (1), A.R.W. Williams (1), J. Piris (1), D.A. Spandidos (2), A.H. Wyllie (1). (1) Department of Pathology, University Medical School, Edinburgh, and (2) Beatson Institute for Cancer Research, Glasgow, Scotland, U.K.

Although the evidence is strong that cellular oncogenes play a role in carcinogenesis, much less is known of their influence on important components of tumour aggression, such as the ability to infiltrate and metastasise. We have studied the behaviour in vivo of early passage fibroblasts, transformed by transfection of the mutated human Ha-ras (T24) gene, under the influence of a strong transcriptional enhancer. In immune-suppressed CBA mice, subcutaneous injection of 10^7 cells usually resulted in progressively-growing, locally infiltrative sarcomas. Lung metastases were detected in 17% of animals and multiple lymph node metastases in a further 17%. No tumour growth, or regression of apparently established tumour nodules, occurred in 22%. Broadly similar results were obtained when the injected cells were from an established (but non tumorigenic) fibroblast cell line, altered by incorporation of either the T24 gene or human c-myc, in the vicinity of transcriptional enhancers. RNA filter hybridisation and immunohistochemical analysis showed that growing tumours expressed the inserted oncogene at high levels and in similar degree to the originally injected cells. Several other oncogenes were expressed at low or undetectable levels only.

We conclude that, if actively transcribed, the mutated c-Ha-ras gene confers competence for metastasis by both blood stream and lymphatics. In these early experiments, high expression of neither c-Ha-ras nor c-myc produced cells which were locally infiltrative but unable to metastasise, or in which blood-borne or lymphatic metastasis was observed in isolation.

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128 IN VITRO INHIBITION OF HUMAN ARTERIAL SMOOTH MUSCLE CELLS PROLIFERATION BY HEPARIN-LIKE GLYCOSAMINOGLYCANS.

R. Tiozzo-Costa, R. Cingi, S. Calandra, M. Barbanti[1] and L. Stanzani[1], Istituto di Patologia Generale, Università di Modena, Modena and Alfa-Farmaceutici, Bologna[1] (Italy).

Recent studies indicate that heparin and heparin-like sulphated glycosaminoglycans may modulate rat smooth muscle cell (SMC) proliferation both in vivo and in vitro. Little is known on the effects of these compounds on the human arterial SMC. Aim of the present study was to investigate the effect of several sulphated glycosaminoglycans, including a mixture of sulphated mucopolysaccharides extracted from pig duodenum (Sulodexide), heparins with different molecular weight and dermatan sulphate on cell proliferation, DNA and protein synthesis of human arterial smooth muscle cells. Arterial SMC were obtained from explants of a 22 weeks human foetus and cultured in vitro. The growth of cells cultured either in the presence or in the absence of glycosaminoglycans (5-100 ug/ml) was monitored from day 1 to day 6. Sulodexide and low molecular weight (LMW) heparins were equally effective in reducing the cell number in a concentration dependent fashion. The greatest growth inhibition (45%) was observed in the 4th day in culture. These compounds delayed the 3-H thymidine incorporation into DNA and shifted the time curve of DNA synthesis. These effects were fully and promptly reversible upon the removal of these compounds from the medium. Protein synthesis was unaffected by these compounds at least quantitatively. Dermatan sulphate (100 ug/ml) was much less effective on cell proliferation than the other glycosaminoglycans tested.

129 INFLUENCE OF INHIBITORS OF THROMBIN ON PORCINE AORTIC SMOOTH MUSCLE CELLS IN PRIMARY CULTURE.

M. Melzig, Christine Harms, E. Teuscher. Department of Pharmacy, Ernst-Moritz-Arndt-University, 2200 Greifswald, Jahnstr. 17, GDR

Enzymatically isolated porcine aortic smooth muscle cells (SMC) were cultivated in M 199 + 15% fetal calf serum. The cells were treated with heparin and two synthetic inhibitors of thrombin: APPA (4-Amidinophenylpyruvic acid-HCl) and NAPAP (N_{α}-(2-Naphthylsulfonylglycyl)-4-amidinophenylalaninepiperididhydroiodide). Heparin and the inhibitors are able to decrease the division rate of SMC and to increase their myosin content. These effects are eliminated by thrombin. The application of inhibitors of thrombin in order to suppress the thrombin induced stimulation of proliferation and of despecialization of vascular SMC and by this means the prevention of arteriosclerotic lesions should be considered.

130 EXPOSURE OF QUIESCENT BP-A31 CELLS TO SERUM PULSES INDUCES THEIR ENTRY INTO THE CELL DIVISION CYCLE INDEPENDENTLY OF PROTEIN SYNTHESIS. H. Gray, T. Buchou, J. Mešter, INSERM U.55, 184 rue du Faubourg Saint-Antoine, 75571 Paris Cédex 12, France.

Chemically (benzpyrene) transformed mouse 3T3 clone A 31 cells (BP-A31) cells become quiescent after 4 days in low-serum (0.05% FCS) in MEM alpha medium. They reenter the cell division cycle when treated with 10% FCS-containing medium, and a sharp peak of ^{3}H-thymidine incorporation is observed at 16-18 h. The amplitude of ^{3}H-thymidine incorporation was a function of the duration of the cell contact with 10% FCS medium, and with a pulse of 2 h, it represented ≃ 15% of that resulting from continuous serum exposure. The mitogenic effect of serum is "registered" by the quiescent BP-A31 cells independently of protein synthesis. When the cells were incubated with the 10% FCS-medium for 2 h in the presence of cycloheximide (CH; 1 µg/ml sufficient to block >90% of protein synthesis) added 1 h prior to serum and removed 1 h after the end of the serum pulse, the ^{3}H-thymidine incorporation peak was delayed by ≃ 3 h but its profile and amplitude were unchanged. CH could be maintained in the culture medium, during and after the serum pulse (2 h), for at least 24 h, without significant reduction of the ^{3}H-thymidine incorporation peak observed ≃ 18 h after removal of CH. Similarly, when longer serum pulses (4 and 8 h) were applied, the presence of CH together with serum delayed the ^{3}H-thymidine incorporation peak by the time corresponding to the duration of the block of protein synthesis. In these experiments, the heights of the peaks observed in CH-treated cells were also reduced, particularly in the 8 h experiment (≃ 50%). Conclusions: 1. Quiescent BP-A31 cells are recruited as a function of time of contact with the mitogens present in FCS; 2. Recruitment is a consequence of translation of mRNA species induced directly (i.e. independently of protein synthesis) by these mitogens; 3. These mRNA(s) are relatively stable and may include that coding for the previously hypothesized "R-protein"; 4. Recruitment itself requires short-lived protein(s).

131 FCS ALSO SUPPORTS THE GROWTH OF PLANT CELLS in vitro

Ferenc Boldog, László Bögre+, Zoltán Marcsek, György Simon, Dénes Dudits+, János Menyhárt. United Research Orgainization of the Hungarian Academy of Sciences and Semmelweis Medical University, H-1082 Budapest, Üllői út 78/a, +:Genetic Institute of the Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary

FCS, a widely known component of a wast majority of media used in the animal cell culture practice, contains an abundance of factors required for maintaining growth of animal cells in vitro. In the experiments presented here the growth supporting ablity of FCS was testeJ in plant cell cultures.

FCS was applied in the concentration range of 1 - 25 % in partially synchronized cultures of lucern cells (line A2) grown in Murashige Skoog medium supplemented with 2-4D and 6-benzyladenine. The effect of FCS was assayed by determining the number (or volume) of lucern cells, the rate of laballed thymidine incorporation and by flow cytometry of protoplasts prepared from the cells used in these expriments. Results obtained in this study revealad that FCS is a potent growth stimulant not only in animal cells but also in plant cells.

132 FACTOR C INCREASES THE K^+ EFFLUX OF Streptomyces griseus MYCELIUM.

F. Szeszák, G. Valu, G. Szabó. Institute of Biology, University Medical School, H-4012 Debrecen, Hungary.

Factor C is a regulatory protein /Mr=30 000/ produced by S.griseus. It can initiate conidium-formation in a mutant of the producing strain.

A method was elaborated for measuring extracellular K^+ in mycelial suspensions of S. griseus by means of an ion-selective membrane electrode. It is suitable for measuring K^+ concentration down to 10^{-6} M under conditions of continuous stirring and aeration.

Upon the addition of factor C an increased K^+ efflux of the mycelium can be observed after 5-10 min latency period. The factor C induced K^+ efflux was characterized in several respects. It can be observed at factor C concentrations down to 0.7 ng/ml and it cannot be prevented by the administration of chloramphenicol or actinomycin D. We tested several other proteins and polymers in our system. No compound had comparable effect to that of factor C. The correlation between the change of K^+ efflux and that of amino acid uptake and protein synthesis was examined.

133 EFFECT OF SODIUM BUTYRATE ON GROWTH, CLONING EFFICIENCY AND TUMORIGENICITY OF KB, MMT AND RPMI CELLS. P. Gálfi, S. Neogrády and F. Kutas. University of Veterinary Sciences, Department of Physiology, H-1400 Budapest, POB 2, Hungary

The growth inhibitory effect of butyrate was investigated in three epithelium-derived cell lines /KB, MMT, RPMI/, on the basis of ^{3}H-thymidine incorporation, cloning efficiency and tumorigenicity. The intracellular distribution of ^{14}C-butyrate was also established to obtain information on the possible causes of dissimilarities in cellular sensitivity to butyrate. The reversibility of inhibition of thymidine incorporation was assessed in MMT cell line. KB, MMT and RPMI cell lines showed a considerable decrease in division rate under the influence of 24-h butyrate treatment. The butyrate doses giving rise to 50 % inhibition of ^{3}H-thymidine incorporation were 2.0, 0.3, 0.2 mM/l, respectively, for the KB, MMT and RPMI cell lines. Examinations with ^{14}C-butyrate have shown that at similar degrees of inhibition of ^{3}H-thymidine incorporation the intracellular butyrate levels were practically identical in all three cell types despite the dissimilarity of the extracellular butyrate concentrations. The ^{3}H-thymidine incorporation of MMT cells exposed to 5 mM/l butyrate for 72 h returned to normal within the next 48 h. By analogy of the butyrate-induced changes in ^{3}H-thymidine incorporation, the RPMI and MMT cell cultures showed greater decrease in cloning efficiency than KB cells. The newborn MMRI mice treated intraperitonically with KB and RPMI cells showed no abnormality, whereas those given MMT cells developed dose dependently several tumors during 6-month observation. Pretreatment of the MMT cells with 5 mM/l butyrate for 72 h accounted for an about 90 % decrease in tumorigenicity. This irreversible decrease was of the same magnitude as that of cloning efficiency.

134 ASPECTS OF THE REGULATION OF CYTOSOLIC pH AND CELL VOLUME

T. J. Rink, Smith Kline & French Research Limited, The Frythe, Welwyn, Hertfordshire, AL6 9AR, U.K.

Non-proliferating cells can maintain a stable cell volume, and well-regulated pH_i, which is typically 1 unit alkaline compared with that expected for electrochemical equilibrium of H^+, for days, weeks or even years. Many mechanisms by which cells recover from relatively large perturbations of volume or pH, will be discussed in this symposium. Changes in pH; in response to mitogenic stimuli, or at fertilisation, have recently been analysed in some detail in several cells; often there seems to be a temporary activation of Na^+/H^+ exchange with consequent alkaline shift of 0.2 to 0.3 units, which may be necessary, but not sufficient, for DNA synthesis, transcription and possibly some translation.

But people only rarely ask what sets volume and what sets the usual pH? Perhaps this is because the honest answer is "we don't know". The conditions for stable cell volume can be baldly stated: (i) no net synthesis of macromolecules; (ii) the net entry or exist of metabolised solutes matches their catabolism or synthesis; (iii) the influx of non-metabolised solutes (e.g. Na, K, Cl) equals their efflux. Little is known of the control of (i). In some cases we can understand (2), e.g. the synthesis of a membrane permeant compound e.g. a steroid hormone is readily matched by its efflux. For (3) we have some detailed information especially for the control of Na, and K. A major threat to cell volume is excessive Na influx: however the activation of pumping as $[Na]^3$ means that within limits increased Ca flux is matched by efflux for only small rises in $[Na]_i$. For pH_i, given mechanisms that set pH near 7.0 the steady state is maintained so long as the acid products of metabolism leave the cell as fast as they are formed. With oxidative metabolism this is simple - the acid is carbonic and the dehydrate, CO_2, is freely diffusible. For glycolysis all one needs is an effective membrane transporter for lactic acid - which most cells have.

Returning to cell volume, I shall briefly discuss 3 aspects: 1, the concept of negative intracellular fluid pressure; 2, the possible mechanism of volume restoration in swollen lymphocytes; 3, the teleology of the astonishing urea transport capacity of the mammalian red cell membrane. (Rink, 1984; J. Physiol. (Paris) 79, 388-394.)

135 REGULATION OF CELL VOLUME AND INTRACELLULAR pH IN EHRLICH ASCITES TUMOUR CELLS: Else K. Hoffmann. August Krogh Institute, University of Copenhagen, 13, Universitetsparken, DK-2100, Copenhagen Ø, Denmark.

When Ehrlich ascites tumour cells are suspended in hypotonic media a regulatory volume decrease (RVD) response occurs which greatly increases both the K^+ and Cl^- permeability of the cells and causes a net efflux of K^+ and Cl^- to decrease the cell volume towards normal values. The chloride permeability (P_{Cl}) is increased to a greater extent than that of K^+, and it is concluded that the K^+ permeability limits the rate of volume change during RVD since volume changes are more rapid when valinomycin or gramicidin is used to provide a parallel pathway for K^+.

The volume change in the presence of gramicidin has been used to monitor the changes in P_{Cl}. P_{Cl} increases abruptly when the cells are swollen but the change in P_{Cl} is transient, with inactivation within about 10 min. The initial increase in P_{Cl} is about 60 fold. Several points of evidence demonstrate that the Cl^- conductance pathway is separate from the K^+ permeation mechanism. The chloride transport pathway has a different anion selectivity and sensitivity to inhibitors than the anion exchange system and the cation,anion cotransport system in Ehrlich cells.

The volume-sensitive pathways can also be activated in isotonic cells; thus addition of the Ca^{2+} ionophore A23187 in isotonic medium induces a fast net loss of KCl both in Ca^{2+}-containing and Ca^{2+}-free media. The net loss of KCl induced by A23187 shows several parallels to the KCl loss induced during RVD, and it is demonstrated that A23187 induces a substantial increase of both the conductive K^+ and the conductive Cl^- permeability. The A23187 induced increase in P_{Cl} in Ca^{2+}-free media (which is probably by release of Ca^{2+} from internal stores) is transient like the volume response, whereas the activation of P_{Cl} is persistent in Ca^{2+}-containing media. These findings suggest that a transient increase in free cytosolic Ca^{2+} may account for the transient activation of the Cl^- transport pathway during RVD. The increase in P_{Cl} is 17 fold after addition of A23187. The potassium permeability increased to a greater extent than that of chloride. The ionophore A23187 plus Ca^{2+}-induced P_K is estimated at 21 fold. The number of Ca^{2+}-activated K^+ channels is estimated at about 100 per cell. A comparison of the A23187-induced K conductance estimated from tracer flux measurements at high external K^+ and from net flux measurements suggests single-file behaviour of the Ca^{2+}-activated K^+ channel involved in RVD. Anti-calmodulin drugs like e.g. pimozide block the volume- or A23187-induced Cl transport pathway as well as the volume- or A23187-induced K^+ transport pathway. It is proposed that the Ca^{2+} activation of both the $K^+$and the Cl^- channel during RVD is mediated by calmodulin.

Preliminary results have demonstrated that calmodulin is decreased in the cytoplasmic fraction and increased in the membrane fraction when Ca^{2+} is present during the preparation of membranes, and A23187 plus Ca^{2+} is added to the cells before isolation of membranes. The mechanism whereby changes in cell volume trigger the permeability increase seems also to involve prostaglandins since the prostaglandin synthesis is blocked during RVD and addition of archidonic acid completely inhibits the RVD response.

Ehrlich cells do not regulate their volume when osmotically shrunken in hypertonic media, but they do show a regulatory volume increase (RVI) if cells were first depleted of K^+,Cl^- and amino acids during RVD and then shrunken by returning them to isotonic media. The net uptake of KCl during RVI is achieved by activation of an otherwise quiescent cation-chloride cotransport with subsequent replacement of

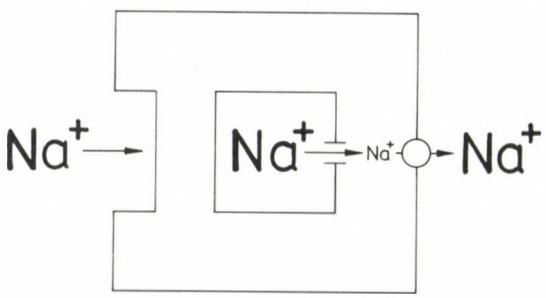
Na^+ by K^+ via the Na^+/K^+ pump. There is a marked increase in the rate of Na^+/K^+ pump activity in the absence of a detectable increase in the intracellular Na^+ concentration. Simultaneous measurements were made of net Cl^- influxes and ^3H bumetanide binding to cells in which the co-transport pathway had been activated during RVI. There was a good linear correlation between inhibition of Cl^- influx during RVI and the number of bumetanide molecules bound per cell, consistent with high specificity of bumetanide binding to cotransport sites. The number of cotransport sites is estimated at $2x10^6$ sites/cell. The turnover-number is calculated at 50 Cl^- ions per site per sec.

Ehrlich cells have pH_i levels that are higher than expected if H^+ and HCO_3^- were passively distributed across the plasma membrane. The internal pH seems to be regulated partly by the Na^+/H^+ exchanger which is inhibited by amiloride and diethylamiloride, and partly by the DIDS-inhibitable chloride-bicarbonate exchange mechanism operating in parallel with a cation-chloride cotransport system. The sodium-coupled chloride influx suffices to increase the ratio of Cl_i/Cl_o above the ratio of $HCO_{3(i)}/HCO_{3(o)}$, providing the necessary conditions for an exchange-coupled influx of bicarbonate ions against their electrochemical gradient. The magnitude of the pH gradient is dependent on external Na^+ which would be the case for both mechanisms. In preliminary experiments we are trying to evaluate the role of the two systems for the control of internal pH mainly by pharmacological evidences.

136 ION TRANSPORT AND VOLUME REGULATION IN Entamoeba histolytica.
Tilly Bakker-Grunwald, Ursula Löhden, Folker Keller, Dorothea Trissl, University of Osnabrück, Dept. of Microbiology, POB 4469, D-4500 Osnabrück, FRG.

Although pathogenicity and immunology of E. histolytica have been the subject of intensive research, surprisingly little is known about the factors that determine ionic composition and cell volume in this organism. Specifically, it has not been established whether the plasma membrane (PM) contains a Na^+ pump, as in animal cells, and/or a proton pump, as in yeasts. To investigate this question we set out to determine the concentrations and fluxes of Na^+ and K^+ in the amoebae.

Much as expected, we found that E. histolytica maintains an ionic steady state in which K^+ is accumulated and Na^+ is depleted compared to the concentrations of those ions in the growth medium (or in a simple saline matching the growth medium in osmolarity and Na^+- and K^+ concentration). However, ion transport measurements were complicated by a constitutively-high pinocytic activity, amounting to the equivalent of up to 30% of the cells' volume per hr. In fact, by employing ^{125}I-polyvinylpyrrolidone as a marker for pinocytic uptake together with $^{22}Na^+$, we established that under normal conditions the trans-PM influx of $^{22}Na^+$ is negligible compared to the rate of pinocytic uptake of the tracer. As the amoebae are in an ionic steady state, they have to get rid of the pinocytically-accumulated Na^+ again. A clue for the pathways involved came from experiments in which we blocked pinocytosis with cytochalasin B. Under these conditions the cells lost Na^+ specifically. Concomitantly they shrunk, and the volume decrease was quantitatively accounted for by the loss of Na^+ together with one other osmotically-active particle (presumably Cl^-). The simplest model to explain these observations (1) is that at least part of the Na^+ taken up pinocytically leaves the cells through an in-series arrangement of a Na^+ "leak", located in the membrane of the pinocytic vesicles (or of secondary vesicles derived from those), with a Na^+ "pump" in the PM. The latter could be either primary-active (e.g., driven by ATP) or secondary-active (e.g., driven by the gradient of another ion). According to this model, the cells would be organized epithelial-like, with the intracellular vesicle compartment corresponding to the lumenal membrane in epithelia.



Model of vectorial Na^+ transport through E. histolytica: a vesicular leak (=) in series with a PM pump (circle).

Our results stress the importance of pinocytic activity and the intracellular membrane compartment involved in this activity for the maintenance of both ionic steady state and cell volume in E. histolytica. However, also for animal cells (2,3) there are indications that intracellular membranes may play a role in cation- and volume homeostasis. In fact, on the basis of stereologic arguments Steinman et al (2) have earlier proposed a model similar to ours for macrophages and fibroblasts.

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2 Steinman, R.M., Mellman, I.S., Muller, W.A. and Cohn, Z.A. (1983) J. Cell Biol. 96, 1-27
3 Russo, M.A., Ernst, S.A., Kapoor, S.C. and van Rossum, G.D.V. (1985) J. Membrane Biol. 85, 1-24

137 VOLUME REGULATION AND Na^+/H^+ EXCHANGE IN HUMAN PLATELETS.
B. Sarkadi, Mária Szirmai, Ilma Szász and G. Gárdos. National Institute of Haematology and Blood Transfusion, 1113 Budapest, Hungary.

Hypoosmotic dilution of human platelets induces a rapid swelling and then a regulatory volume decrease /RVD/ in these cells. RVD is inhibited by quinine, oligomycin, trifluoperazine and N-ethylmaleimide, whereas it is unaffected by ouabain. In high K^+ media hypoosmotic dilution is not followed by RVD but rather by a further volume increase. This secondary volume increase is greatly facilitated by gramicidin while blocked by trifluoperazine or oligomycin. External EGTA /1 mM/ blocks the normal RVD reaction or the secondary swelling in high K^+ media. According to these results human platelets possess a volume regulatory mechanism similar to that of lymphocytes, that is the opening of volume-induced K^+ and Cl^- transport pathways in hypoosmotic media.

In human platelets the presence of a Na^+/H^+ exchange is indicated by a rapid swelling in a Na-propionate medium. This phenomenon is based on the influx of undissociated propionic acid and a following Na^+ entry through a Na^+/H^+ exchange pathway. The process is amiloride-sensitive and is greatly facilitated by the addition of the calcium ionophore A23187. A facilitating effect on this swelling is also observed upon the addition of thrombin or phorbol esther /TPA/. The detailed analysis of the volume-, and pH-dependent ion transport pathways may help in the further understanding of the reactions involved in platelet activation under physiological or pathological conditions.

138 BIOCHEMICAL AND PHARMACOLOGICAL PROPERTIES OF THE Na^+/H^+ ANTIPORT OF EUKARYOTIC CELLS. ITS ROLE IN pHi REGULATION AND VOLUME REGULATION.
Michel Lazdunski, Christian Frelin, Paul Vigne, Thierry Jean, Olivier Chassande. Centre de Biochimie du CNRS, Université de Nice, Parc Valrose, 06034 Nice Cedex, France.

Most eukaryotic cells possess a membrane structure that catalyses the electroneutral exchange of Na^+ for H^+. This system is inhibited by amiloride and its more potent N-5-disubstituted derivatives. The biochemical properties of the Na^+/H^+ exchanger were studied in skeletal muscle cells, cardiac cells, fibroblasts, rat brain synaptosomes and glial cells using a combination of the $^{22}Na^+$ uptake experiments, H^+ efflux experiments and pHi measurements. The system plays an important role for the control of internal pH in fibroblasts and skeletal muscle cells. In chick cardiac cells, the Na^+/H^+ exchanger serves as an uptake system for Na^+ using the transmembrane H^+ gradient that is maintained by other pHi regulating mechanism. The properties of interaction of the antiporter with external Na^+ and H^+ and with internal H^+ were studied in detail. In all systems internal H^+ interacts cooperatively with the Na^+/H^+ antiporter. In rat brain synaptosomes, the internal pH dependence of the Na^+/H^+ antiporter is also dependent on the value of the external pH.

In undifferentiated myoblasts, the antiport can be activated by serum growth factors, epidermal growth factor and phorbol esters. This activating effect is lost when cells have differentiated into myotubes. In rat myotubes, the Na^+/H^+ exchanger is present in an already activated state. Epidermal growth factor and phorbol esters seem to produce an activation of the Na^+/H^+ exchanger in myoblasts by distinct routes that may involve a phosphorylation of the antiporter.

An activation of the Na^+/H^+ antiport was observed in glial cells following an osmotic cell shrinkage. The mechanism of activation will be described.

A [^{3}H]labelled derivative of amiloride : ethylpropylamiloride was prepared and used to characterize the amiloride binding site of the Na^+/H^+ exchange system in kidney and rat brain synaptic membranes. This derivative appear to be very useful for the purification of the Na^+/H^+ antiporter.

139 REGULATION OF CYTOPLASMIC pH IN QUIESCENT AND ACTIVATED POLYMORPHONUCLEAR LEUKOCYTES.
S Grinstein, W Furuya, PE Nasmith and WD Biggar. Research Institute, The Hospital for Sick Children, Toronto, Canada.

Proton equivalents tend to accumulate in the cytoplasmic compartment of quiescent neutrophils due to the transmembrane potential and to the continuous generation of acid by anaerobic metabolic pathways. However, regulatory mechanisms maintain the cytoplasmic pH (pH_i) in the physiological range ($\sim$ 7.1). The nature and properties of these mechanisms were investigated using electronic cell sizing and the fluorescent pH_i indicator bis(carboxyethyl)carboxyfluorescein. Cells were artificially acid-loaded using weak acids or ionophores and pH_i was measured in different media. Addition of external Na^+ (Na^+_o) to acid-loaded cells resulted in intracellular alkalinization due to transmembrane H^+ (equivalent) flux. The alkalinization was associated with Na^+ uptake and both processes were blocked by amiloride, suggesting Na^+/H^+ countertransport. The rate of amiloride-sensitive H^+ efflux could be calculated from the rate of change of pH_i, using a buffering power of 28 mmole.liter^{-1}.pH^{-1}, determined by titration with NH_4^+ or propionate$^-$. The rate of Na^+_o/H^+_i exchange was a saturable function of extracellular Na^+ (apparent K_m = 73 mM). Forward (Na^+_o/H^+_i) exchange was inhibited by elevating external [H^+] or internal [Na^+] and competitively by amiloride (apparent K_i = 24 µM). The antiport was virtually inactive in unstimulated cells at $pH_i \geq 7.2$, but was markedly stimulated by cytoplasmic acidification, consistent with a role in pH_i homeostasis.

When treated with 12-O-tetradecanoylphorbol 13,acetate (TPA), normal neutrophils undergo a biphasic change in pH_i: an initial acidification is superseded by a moderate alkalinization. The latter reflects activation of Na^+/H^+ exchange inasmuch as it requires extracellular Na^+ and is inhibited by micromolar concentrations of amiloride. The initial acidification, which is fully expressed in Na^+-free media, is accompanied by efflux of H^+ equivalents, suggesting a metabolic origin. Four lines of evidence suggest that the cytoplasmic acidification is associated with superoxide synthesis and/or the attendant stimulation of the hexose monophosphate shunt: 1) Inhibition of these pathways by N-ethylmaleimide, iodoacetamide or deoxyglucose prevents the acidification; 2) When neutrophils are activated by a variety of soluble stimuli in Na^+-free medium, the rate and magnitude of oxygen consumption are proportional to the rate and magnitude of the intracellular acidification; 3) Activation of the hexose monophosphate shunt in resting cells by oxidation of NADPH with phenazine methosulfate or methylene blue is associated with cytoplasmic acidification; 4) Neutrophils from patients with chronic granulomatous disease, which are unable to generate superoxide and other activated oxygen metabolites, failed to acidify their cytoplasm in response to phorbol esters. The results indicate that large amounts of H^+ (equivalents) are generated metabolically in the cytoplasmic compartment of activated neutrophils. However, pH_i is maintained constant, and can even become slightly alkaline due to the operation of a pH_i homeostatic system: the Na^+/H^+ antiport.

140 *DIFFERENTIATION OF HL-60 CELLS INDUCED BY RETINOIC ACID IS ACCOMPAGNIED BY AN INCREASE IN INTRACELLULAR pH.*
A. Ladoux[1], C. Frelin[2], B. Geny[1], E.J. Cragoe Jr[3], J.P. Abita[1]. 1 : Unité 204 INSERM, Hôpital St Louis 75475 PARIS CEDEX 10, FRANCE. 2 : Centre de Biochimie du CNRS, Parc Valrose, 06034 NICE CEDEX, FRANCE. 3 : Merck Sharp and Dohme Research Laboratories, West Point, Pennsylvania 19486, USA.

The human promyelocytic leukemic HL-60 cells undergo a differentiation program after treatment by retinoic acid (RA). The cell volume decreases from 800 µm^3 to 500 µm^3 and the cells acquire typical granulocytic properties.

The internal pH of HL-60 cells was measured either from the distribution of [^{14}C] Benzoic acid or from the fluorescence of intracellularly-trapped bis-carboxyethylcarboxyfluorescein (BCECF). During differentiation of HL-60 cells by RA an increase in the pH_i from 7.03 ± 0.05 to 7.27 ± 0.03 is observed after five days of culture. HL-60 cells from a clone resistant to RA-induced differentiation have the same pH_i when cultured in the absence or in the presence of 0.5 µM RA.

At least two different mechanisms regulate the pH_i in HL-60 cells : i) a Na^+/H^+ exchange system which is active when $6.2 < pH_i < 7.8$ and ii) a Na^+ dependent Cl^-/HCO_3^- exchange system. The Na^+/H^+ exchange system is specifically inhibited by amiloride and its more potent derivatives dimethyl and ethylisopropyl amiloride. Both systems catalyze the influx of $^{22}Na^+$ into HL-60 cells. Differentiation of HL-60 cells by RA is accompanied by a two fold activation of the Na^+/H^+ exchange system. By contrast the activity of the Na^+ dependent Cl^-/HCO_3^- exchanger remains constant during differentiation. Activation of the Na^+/H^+ exchanger is observed at all pH_i values between 6.2 and 7.8.

These results suggest that in HL-60 cells, RA produces an activation of the Na^+/H^+ exchanger which, then, leads to the observed cellular alkalinisation.

141 CHANGING OF SURFACE ANTIGENS ON MEMBRANE OF PARAMECIUM PRIMAURELIA.
C. ANTONY and Y. CAPDEVILLE.
Centre de Génétique Moléculaire. CNRS. 91190 GIF-SUR-YVETTE. FRANCE.

The surface antigens of Paramecium primaurelia constitute a thick protein coat covering the whole surface of the prtozoan. These molecules belong to a large family of high molecular weight iso-proteins whose alternative expression is conditioned by environmental factors such as temperature, medium composition etc. Tracking of surface antigens during replacement induced by temperature shift is being accomplished using electron microscopic immunolabeling. Specifically, double labeling of the new and old antigen types is performed with corresponding antibodies, directly coupled with gold probes. Using this method we are describing the appearance of the new antigen and the elimination of the one previously expressed at the surface of the membrane.

142 THE EFFECT OF BIOLOGICALLY ACTIVE MATERIALS ON THE EXPRESSION OF LYMPHOCYTE CELL SURFACE MARKERS
Dea Kotlán/1/,Günther Böck/2/,Éva Rajnavölgyi/3/,Christopher Huber/4/,Győző Petrányi/1/ /1/ nat Inst of Haematology and Blood Transf,1113 Budapest, Daróczi 24,Hungary,/2/ Inst for Gen and Exp Pathology,Univ of Innsbruck Med School, A-6020 Innsbruck,Fritz-Pregl 3,Austria /4/ Univ Clin of Medicine, A-6020 Innsbruck,Anich str 35,Austria /3/ ELTE dep of Immunology,2131 Alsógöd Jávorka 24, Hungary

The current work investigates the effects of interferons/IFN/, interleukins/IL/, prostaglandins/PGE/ and other biologically active materials on the expression of hystocompatibility and differentional antigens/Ag/ of lymphocytes. The methods used,are:indirect RIA,Immunofluorescence assay-FACS analysis and ELISA. It has been demonstrated, that crude, purified or recombinant α and γ IFNs increase the expression of HLA ABC,β_2m antigens after 16 hours' incubation. The amount of HLA DR Ag doubled following a few hours' γ IFN treatment, while αIFN was not effective. Among the T4,T8,T3, T11 differentional Ags, only the T11/CD2/ Ag's expression increased after a few or more hours' IFN incubation. Purified IL-2 enhanced strongly the HLA DR and β_2m Ags' amount, but in the case of differentional Ags except the T11 Ag it did not seem to be effective. The expression of Leu 7 Ag didn't change either after interferons or interleukins. After about 6 hours' incubation with PGE_2,PGF_2 the amount of HLA DR Ag dicreased slightly. On the basis of the results up to now, it can be concluded, that biologically active materials have very different effects on the cell surface antigens' expression.

143 MONOCLONAL ANTIBODIES TO THE SURFACE ANTIGENS OF HUMAN AORTA SMOOTH MUSCLE CELLS
M.A. Glukhova, A.Y. Kabakov, O.I. Ornatsky, M.G. Frid. Institute of Experimental Cardiology, USSR Cardiology Research Center, Academy of Medical Sciences, 3rd Cherepkovskaya Str. 15A, 121552, Moscow, USSR

A panel of monoclonal antibodies recognising a surface of cultured human aorta smooth muscle cells (SMC) was generated. One of the antibodies (IIG10) was shown do not bind to endothelial cells from aorta and umbilical veen. An antigen recognized by the antibody has a molecular mass of 330000 as determined by immunoprecipitation. Immunoperoxidase staining with IIG10 antibody reveals the antigen in the section of human aorta, thus the expression of the antigen is characteristic of SMC in situ and does not result from the cultivation. The same antigen appeared to be present on the fibroblast surface while neither immunofluorescense and flow cytofluorimetry nor immunoprecipitation reveal it in endothelial cells. Distinguishing between SMC and endothelial cells antibody IIG10 may serve a vector for directed transport of drugs to denuded areas of blood vessel wall where subendothelium is exposed to circulation. In order to prove that we used an in vitro model system: erythrocytes precoated with secondary (antimouse Ig antibodies were let to interact with cocultivated SMC and endothelial cells pretreated with antibody IIG10. Under the conditions of experiment antibody IIG10 provided an absolute diserimination erythrocytes-potential containers for carrying the drugs, bound only to SMC, while the surface of endothelial cells retained clear.

144 CELL SURFACE CARBOHYDRATE ALTERATIONS AND LECTIN EFFECTS ON CELLULAR BEHAVIOR.
FONT J., DOYENNETTE M.-A., CODOGNO P. AND AUBERY M. Unité INSERM 180, CNRS UAC 81, 45-rue des Saints-Pères, 75006 PARIS, France.

The modifications induced by tunicamycin (TM), a specific inhibitor of the biosynthesis of N-glycans, on concanavalin A (Con A), Ricinus (RCA 120) and Peanut (PNA) lectin bindings in cultured fibroblasts derived from chick embryos were investigated, in parallel to the possible alterations of lectin effects on cell behavior. In the case of Con A, TM induced a slight but significant decrease (30 %) of the number of lectin binding sites, whereas the affinity constant remained unchanged. The effect of Con A on cell growth was not altered after TM treatment of the embryo cells, and the remaining N-glycosylated sugar chains present at the cell surface might be sufficient for the biological effect of Con A. In the case of RCA 120, TM treatment of embryo cells increased (2-fold) the number of both the high and the low affinity binding sites. The affinity of each class of binding sites was decreased (30-35 %) by TM treatment. In parallel the lectin toxicity was increased (2.8 fold) as determined by ^{3}H-leucine incorporation. In this case, when the N-linked glycoprotein synthesis was inhibited, glycoproteins with O-linked carbohydrate chains and/or glycolipids might take place as useful functional RCA 120 binding sites. The newly available binding sites containing a Gal-GalNAc sequence were identified by the binding of PNA, the site number of which increased 9-fold after TM treatment of embryo cells. Also, the remaining N-linked glycoproteins present at the cell surface after TM treatment of embryo cells might be active binding sites for RCA 120.

145 BINDING AND ENDOCYTOSIS OF CONA IN ACANTHAMOEBA DURING DIFFERENT GROWTH PHASES
Gun Paatero, Curt West, Lilian Wikström, Boris Isomaa. Department of Biology, Abo Akademi, SF-20500 Turku, Finland

Previous work has shown that the ConA-mediated agglutination in Acanthamoeba castellanii decreases during the logarithmic growth phase and is markedly reduced in the transition to the stationary growth phase (Isomaa et al., Eur. J. Cell Biol., in press). The purpose of the present study was to determine whether there are growth phase dependent alterations in the binding of ConA to the plasma membrane and in the distribution of ConA receptors in Acanthamoeba.

The binding of [^{3}H]ConA-ConA (1 µg/ml) at 4°C and 28°C to amoebae in the logarithmic and early stationary growth phase did not show growth phase dependent variations. Exposure of amoebae to TRITC-ConA at 2 µg/ml and 10 µg/ml for 4 min and 15 min at 28°C displayed a staining pattern characterized by a diffuse cell surface distribution and by a various number of distinct small aggregates. The aggregated staining pattern was increased with time and at the higher ConA concentration cap-like structures were frequently observed in amoebae in the logarithmic growth phase, but not in amoebae in the early stationary growth phase. In order to distinguish intracellular ConA from ConA bound to the cell surface a double-label immunofluorescence technique was used. The results show that the distinct fluorescent aggregates and the cap-like structures represent intracellular ConA. Electron microscopic observation of ferritin-ConA labelled amoebae confirms the results of the immunofluorescence experiments. Furthermore, internalization of ConA is also indicated by time course studies using [^{3}H]ConA. Thus, the growth phase dependent alterations of the ConA-mediated agglutinability apparently do not reflect changes in the binding of ConA to the cell surface but changes in the dynamic properties of the plasma membrane of Acanthamoeba.

146 MODIFICATION OF CELL SURFACE WITH POLYANIONS AND POLYCATIONS - BINDING OF FLUOROCHROME /CDC/ AS A MARKER OF MEMBRANE HYDROPHOBICITY
Elżbieta Wyroba[1], Giovanni Bottiroli[2], and Paolo A. Giordano[2]. /1/ Department of Cell Biology, Nencki Institute of Experimental Biology, 02-093 Warsaw, Poland and /2/ Centro di Studio per l'Istochimica del C.N.R., 27100 Pavia, Italy.

Polycationic poly-L-lysine and polyanionic poly-L-glutamic acid have been used as the agents modifying the cell membrane of the living protozoan cell Paramecium aurelia. The treated cells have been subsequently labelled with cycloheptaamylose-dansyl chloride complex /CDC/ shown previously to be an useful fluorescent marker of surface membrane /Histochemistry 73, 459, 1981 and ibid 77, 171, 1983/. The microspectrographic analysis of CDC-derived membrane fluorescence was performed using multichannel analyzer. The results indicate that spectral shape of emission curves of CDC-labelled cells treated with polyanion or polycation was identical as in the controls but the position of the maximum was significantly shifted to the longer wavelength in the poly-L-lysine modified ciliates. This is the characteristic feature of less hydrophobic microenvironment of fluorochrome molecules. The experiments performed on the solutions of lysine, poly-L-lysine and CDC and their mixtures at different ratio exclude the possibility that the observed effect may be due to the interaction between the molecules of the applied chemicals /polycation and fluorochrome/. Thus it is suggested that poly-L-lysine treatment increases the membrane hydrophilicity and this alteration may be monitored by microspectrographic analysis of the CDC bound subsequently to such a pretreated membrane.

147 SURFACE CHARGE DISPERSION OF THE EGGS AND EMBRYOS OF THE SOW AND MOUSE.

I. Veres /1/, L. Mettler /2/, R. Gimes /3/, K. Elze /4,/, A. Czuppon /2/, M. Schnurbusch /4/, B. Nagy /3/, É. Mezlényi–Láncz /1/, F. Csillag /5/, A. Öcsényi /1/. /1/ Univ. Agric. Sci. Gödöllő, Hung. /2/ Univ. Frauenklinik, Kiel GFR, /3/ Clinic of Gynecology, Medical School, Budapest, /4/ Vet. Med. Reproduction Univ. Leipzig, GDR, /5/ Univ. Horticulture, Budapest.

It was already earlier developed a new method by one of us for study of the dispersion of surface charges of the very different living cells used AgI colloid label sol in TEM and SEM.

We killed the non fertiliezed resp. fertilizied animals and we prepared eggs resp. embryos. We labeled fresh resp. with GA prefixed objects used positive resp. negative charged AgI sols. We studied them directly in SEM and by means of ultrathin sectioning after postfixation with OsO_4 in TEM.

The eggs and embryos were showing a net negative surface charge, however the density of the anionic sites was lower as that of the spermatozoa of boar and mouse and it seemed to be statistical equally, random–like dispersed. However in the case of the egg of sow there were labeled al ways only one of the sides of the fibres of the Zona pellucida.

There is a possible way of the interpretation: the adhesion of the negative charged spermatozoon may be supported by means of the lack of negative charges in micro–areas of the Zona.

We want to study also the semiquantitative charge dispersions following the cell differentation used biometrical evaluation, too.

148 SURFACE CHARGES OF THE REPRODUCTIVE CELLS. /SPEMATOZOA, EGGS AND EMBROYS./

I. Veres. EM– Central Lab. Univ. Agric. Sci. H–2100 Gödöllő, Páter K. u.1.

The surface charges of the very different mammal, bird and fish spermatozoa resp. that of eggs and fertilizied eggs were studied in TEM, SEM and microanalysis used Silver Iodid and Ferritin label techniques and Lanthanum resp. Ruthenium Red stains. /Human, mouse hamster, rabbit, cattle, swine, wild swine, deer, roe deer, turkey, goose, etc. and fish cells./

It was stated the net negative surface of the spermatozoa and the different charge densities. There are always charge concentrations toward the middle–, principal–, and especially toward the end–pieces assuring the collision's –free swimming of the cells on this way.

It was also stated the negative surface charge of the eggs and of the fertilizied eggs but the charge holders are showing randomlike distributions there and their densities are lower as measured on the spermatozoa.

There were also stated the dramatical changes of the surface charges of mammal spermatozoa after the acrosome reaction: The density of the negative charge holders is decreased and some positive charged components are also appearing in the Acrosome complex. Actually we are starting paralell investigations concerning also the lectin–binding capacity of the objects studied by us. The complex evaluation of the label–techniques seems to be very useful assuring new biophisical–biochemical informations about the Glycocalyx.

The findings are supporting biomedical and veterinar projects. /Testing, cryoconvervation, insemination, transplantation etc./

149 IMMUNOCYTOCHEMICAL STUDIES ON THE BINDING OF MICROSOMAL ANTIBODIES TO THYROID FOLLICLE CELLS

Mikael Nilsson (1), Johan Mölne (1), F. Anders Karlsson (2), Gunnar Engström (3), L.-E. Tisell (3), Lars E. Ericson (1). (1) Dept. of Anatomy, University of Göteborg, (2) Dept. of Internal Medicine, Uppsala, (3) Dept. of Surgery I, Sahlgrenska Hospital, Göteborg, Sweden

Antibodies to thyroid microsomal antigen are present in sera of patients with autoimmune thyroid diseases. The binding of microsomal antibodies (Msab) to thyroid follicle cells was studied by immunocytochemistry. Open follicles were prepared by collagenase digestion of human thyroid tissue, obtained at surgery for toxic goiter. The follicles were incubated for 60 min at 4°C with sera with high titres of either Msab or thyroglobulin antibodies (Tgab) and then for 30 min at 4°C with protein A-colloidal gold or rabbit anti-human IgG-colloidal gold. Follicle samples were also further incubated for 30 min at 37°C with or without addition of TSH. The follicles were then fixed and processed for electron microscopy.

Follicle cells in newly isolated, open follicles retained their polarity. The cells responded to TSH by forming pseudopods projecting from the apical cell surface. No gold particles were associated with follicles incubated with normal serum or serum containing Tgab. After incubation with Msab gold particles were selectively located on the outer surface of the apical plasma membrane; practically no particles were found on the basolateral plasma membrane. All cells were labelled although the concentration of gold particles varied. In cells kept at 4°C the gold particles were more concentrated on microvilli than on smooth portions of the apical plasma membrane. In follicles incubated at 37°C the gold particles frequently formed clusters, often associated with coated pits and they were also present in deep plasma membrane invaginations and in vesicles and lysosomes in the apical cytoplasm. No particles were found on the membrane of TSH-induced pseudopods.

These observations indicate that microsomal antigen is a constituent of the apical plasma membrane. The antigen-antibody-colloidal gold complex is internalized by micropinocytosis but not by macropinocytosis.

150 NON-RANDOM DISTRIBUTION OF EPIDERMAL GROWTH FACTOR RECEPTORS ON THE SURFACE OF A431 CELLS.
Maria Rosaria Torrisi, Antonio Pavan, Lavinia Lotti, Claudia Zompetta, Alberto Faggioni and Luigi Frati. Dipartimento di Medicina Sperimentale, Università degli Studi "La Sapienza", Roma, Italy.

Immunoelectron microscopic analysis of Epidermal Growth Factor (EGF) receptor distribution over the plasma membrane of A431 cells has been performed using surface replica technique as well as conventional thin sections. The immunolabeling was obtained with an anti-EGF receptor monoclonal antibody (Oncor, Gaithesburg, Md, USA) followed by Protein A-colloidal gold conjugates. The distribution of the immunolabeling in thin sections was clearly not uniform, but revealed a preferential concentration in the areas of the microvilli. Similar results were obtained using surface replica technique, which allows labeling and observation of the surface plane of the plasma membrane of cells growing on coverslips. Here again a non-random distribution of the labeling with concentrations on the microvilli and vesicle areas and at the free margin of the cells was found. The same pattern of distribution was observed on human KB cells and murine Balb c/3T3 cells. Since this type of distribution has been reported for recycling receptors such those for transferrin on A431 cells, our observations suggest that this distribution is not peculiar of recycling receptors, but characteristic of EGF receptors as well.
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151 IMMUNO-ELECTRONMICROSCOPICAL LOCALIZATION OF Fc RECEPTORS IN HUMAN NEUTROPHILS.
C.R. Jost (1), J.A.M. Fransen (1), R. de Goede (1), P.A.T. Tetteroo (2), M.R. Daha (3), L.A. Ginsel (1). (1) Laboratory for Electron Microscopy, and (3) Department of Nephrology, University of Leiden, Rijnsburgerweg 10, 2333 AA Leiden, (2) Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology, University of Amsterdam, The Netherlands

Immune complexes are cleared from circulation by phagocytes, i.e. granulocytes, monocytes and macrophages. For this purpose the plasma membranes of phagocytes contain Fc receptors (FcR). We developed immuno-electronmicroscopical methods to study the intracellular transport of both the FcR and of immune complexes in human neutrophils. This abstract deals only with the localization of the FcRs. For this purpose we used a monoclonal antibody against the neutrophil FcR, CLB-FcR-gran 1. It was possible to label the FcR in unfixed neutrophils using CLB-FcR-gran 1, a rabbit polyclonal antibody against mouse IgG and protein A-gold respectively. However, it proved impossible to label the FcR in Lowicryl K4M and cryosections of 1% paraformaldehyde (PFA), 0.1% glutaraldehyde (GA) fixed material using a similar labeling procedure. Therefore other fixatives were tested using radio-immunoassays. The cells were incubated with CLB-FcR-gran 1 and goat anti-mouse I^{125} respectively. From the data presented in the table we conclude that only paraformaldehyde and periodate-lysine-1% PFA (PLP) are suitable fixatives. Immunogold-labeling of 1% paraformaldehyde-fixed material, indeed showed FcR labeling on the plasma membrane and possibly in phago-lysosomes. The preservation of the ultrastructure of the cells in the cryosections is considerably improved by using 1% paraformaldehyde in sodiumbicarbonate buffer instead of in Sørensen phosphate buffer.

fixative	cpm + sd
non	2666 ± 51
0.2% PFA	2533 ± 224
0.5% PFA	2912 ± 109
1% PFA	3019 ± 118
PLP	2726 ± 77
0.05% GA	163 ± 66
0.1% GA	177 ± 51
0.1% acrolein	281 ± 9
1% PFA and 0.1% GA	160 ± 18
1% PFA and 0.1% acrolein	297 ± 7

152 PERIODATE OXIDIZED ADENOSIN DIPHOSPHATE INHIBITS THE ADENOSIN DIPHOSPHATE INDUCED PLATELET AGGREGATION.
Albertino Ferri(1), Paola Conigli(1) and Ugo Taddeo(2). (1) Istituto di Chimica Biologica, Università di Ferrara I-44100 Ferrara, via L.Borsari 46, and (2) Clinica di Patologia Speciale Medica, Università di Ferrara, I-44100 Ferrara, C.so Giovecca, Italy.

Adenosin diphosphate (ADP) is the most important trigger of platelet aggregation: first inducing a change in the platelet shape from discs to spiculated spheres and subsequently by promoting platelet association and storage granules secretion. The mechanism by which ADP triggers the platelet functions is still unknown. Because of its negative charges it seems unlikely to penetrate inside the cell across the membrane, more plausible hyphotesis seems to be that a membrane protein may act as the ADP receptor capable to transmit the signal into the intracellular system. Here we present a preliminary study on the interaction of periodate oxidized ADP (o-ADP) with platelets in human platelet rich plasma (PRP). PRP samples ($2.5x10^8$ cells/ml) incubated up to 30 minutes either at R.T. and at 37°C under continous stirring with various concentration of o-ADP (up to 1mM) did not undergo to aggregation. o-ADP has assaied as inhibitor of the aggregation induced on platelets by 2uM ADP and showed a concentration dependent inhibitory effect that reached about 80% when it was 0.3 mM. In order to investigate wether o-ADP competes with ADP to bind on the platelet membrane we measured the binding of ^{14}C-ADP on platelets preincubated with increasing concentrations of o-ADP (5 minutes, 37°C in rest) and we found a decrease in labeled ADP binding parallel to the decrease of the aggregation of platelets. This is a preliminary indication taht o-ADP may be an inhibitor acting via a competitive binding to the receptorial sites for ADP on the platelet membrane.

153 DEMONSTRATION OF SURFACE RECEPTORS ON MACROPHAGES BY MEANS OF SEM.
K. Augsten(1), T. Peschke(1), A. Gabert(2). (1)Academy of Sciences of the GDR, Central Institute of Microbiology and Experimental Therapy, Jena, Beutenbergstraße 11, and Institute of Biotechnology, Leipzig, Permoserstraße 15
Gold-labelling of surface receptors is used to investigate functional processes of cells, e. g. endocytosis, immunological response or interactions between cells, etc. The labelling was performed on unfixed and variously fixed murine peritoneal macrophages (PMa) and the staining efficiency (signal to noise ratio) was evaluated by means of fluorescence microspectrophotometry and X-ray microanalysis. The single PMa was imaged at magnification of about 10 000x in a JEOL JSM-35 SEM, and the elemental content was determined with a Kevex X-ray energy spectrometer at standardized area of analysis. Additionally, the morphological appearance of PMa was evaluated by means of SEM in dependence on the kind of fixation and the labelling procedure. Under defined experimental conditions it is possible to determine semiquantitatively the amount of Au of such labelled ConA-receptors on PMa. The influence of the fixatives formaldehyde (FO), glutaraldehyde (GA), and OsO_4 on the amount of Au-labelled ConA-receptors was determined and compared with the results of fluorescence microspectrophotometry after labelling with FITC-ConA. The results from the X-ray microanalysis have shown that fixation with 1% FO in PBS provides the best results. OsO_4 proved to be least suitable. GA was a bit worse than FO, but it was the best fixative for maintaining the fine structure of the PMa. These findings are in agreement with those obtained by fluorescence-photometry. When the FITC-ConA-labelling was carried out after fixation, the unspecific fluorescence intensity was very high for OsO_4 but not detectable in case of GA-fixed cells. These results were reverse when fixation preceded labelling.

154 TRANSFER OF RECEPTORIAL HORMONAL IMPRINTING TO NON IMPRINTED CELLS.
Péter Kovacs, György Csaba, Ottilia Török, Bálint Madarasz, Edit Bohdaneczky. Dept. of Biology, Semmelweis Univ. of Med. Budapest, Hungary
Fletcher and Greeman observed at 1985, that less receptor bearing cells bind hormone, than will be activated in cell culture and supposed the information transfer /likely through gap junctions/ between the cells. As hormonal imprinting is also a first of all receptor level event, in the present experiments the transfer of imprinting had been studied in Chang liver and Chinese hamster ovary /CHO/ cell line cultures using insulin and TSH or FSH-LH, respectively, as imprinting provoking hormones. For the detection of results FITC-labelled - adequate - hormones had been used, and the cell contacts had also been studied /electronmicroscopically/ by using colloidal gold labelled insulin. The experiments cleared that hormonal imprinting could be transferred by cell contact, as mixture of imprinted and unimprinted cells showed similar mean binding capacity, as cells of totally imprinted cultures. As a route of information transfer gap junctions are not likely, but many insulin accumulated between cell processes in coated pits.

155 A SPECIAL TYPE OF GAP JUNCTION AS ZONULA-OCCLUDENS-LIKE STRUCTURE IN ODONTOBLASTS
H.Hörandner, H.R.Ronge, W.Pritz Institut für Mikromorphologie der Univ. Wien
Odontoblasts constitute an epithelial-like layer of columnar cells originating from mesenchyme and situated at the inner surface of teeth. In thin sections these cells appear closely apposed at their apices with some membrane condensations. Cell junctions, however, are not easily discerned. In freeze-fractured specimens, the junctional region, situated about 2µm below the osteoblasts dentinal processes, appears as a belt about 1µm wide and made up by an irregular meshwork of strands. The mesh size is around 0,1µm and a single strand consists on the P-face of a row of 11nm particles with varying interspaces and frequently intercalated clusters of about 10 to 50 densely packed particles, and the corresponding pits in the E-face. Immediately below this beltlike junction are situated large plaqueform gap junctions up to 2µm in diameter. the remaining cell membrane extending for 60 to 70µm to the cell base is covered with innumerous small gap junctions mostly between 0,1 and 0,5µm in diameter. Small cytoplasmic processes, about 0,25µm in diameter (presumably free nerve endings) lying in between the large cell bodies of the odontoblasts and extending up to the predentin zone are usually accompanied by 1 or 2 rows of particles or pits, similar to those in the beltlike junction. Whether this specialized cell junction type is due to the odontoblasts unability to form true tight junctions or to the need for a special paracellular permeability, is not known at present.
Reticular gap junctions have been found in various tissues, however not in this continuous beltlike arrangement.

156 A QUANTITATIVE ELECTRON MICROSCOPE ANALYSIS OF GAP JUNCTIONS IN RAT ANTRAL FOLLICLES. Dominique Raick, Edgard Baeckeland. University of Liège, Service Embryology B-4020 Liège, Belgium.

The control mechanism for mammalian oocyte maturation is one of the major puzzles of developmental biology. The intrafollicular environment inhibits this maturation. By an unknow mechanism LH overcomes this inhibition and stimulates oocyte maturation in vivo.

A small polypeptide called OMI (Oocyte maturation inhibitor) and cAMP have been proposed as inhibiting substances synthetized in the granulosa cells and transmitted to the oocyte in order to maintain meiotic arrest.

The network of gap junctions connecting the granulosa cells and the cumulus cells with the oocyte appears to be critical for the regulation of oocyte maturation. Thus we decided to study junctions in the different regions of the follicle during folliculogenesis prior to ovulation from a quantitative and ultrastructural point of view. Lanthanum tracer was used to delineate the gap junctions for the morphometrical study. Our results concerning the antral stage suggest that the relative area of gap junctional membrane is greatest in the cumulus and peripheral regions of the follicle than in the antral region.

Therefore, we must intend that the breaking of communication preceding the ovulation may occur in the antral region rather than between oocyte and cumulus cells. This hypothesis must be confirm by the study of the stages following the LH surge.

157 CELL COMMUNICATION COMPARTMENTS IN MOLLUSCAN EMBRYOS. F. SERRAS and J.A.M. VAN DEN BIGGELAAR. Zoological Lab. Univ. of Utrecht. The Netherlands.

Embryonic cells are capable to develop pathways of cell communication, which may allow the passage of regulatory molecules involved in the control of development. Ultrastructural studies have shown the presence of gap junctions as a morphological unit between interconnected embryonic cells. We report here that the restrictions of gap junctional permeability in mosaic embryos, reflects the presence of boundaries between regions with different developmental fates. We have iontophoretically injected Lucifer Yellow in embryos of Lymnaea stagnalis, to visualize the interconnected cells. From the four-cell stage to the 24-cell stage all cells are mutually communicated. At the 48-cell stage appears the first indication of restrictions in cell communication. At this stage four groups of cells, which later will raise to a ring of larval cells, become uncoupled from the rest of the embryo. Later on, dye-couping is progressively restricted within groups of cells with specific developmental fates. We describe the formation of cell communication compartments in association with the formation of developmental compartments.

158 **DYNAMICS OF A TRANSIENT COUPLING BETWEEN ER PARACRYSTALS AND PLASMA MEMBRANE.** J.M.Bassot(1), M.Girard(2), M.T.Nicolas(1) and G.Nicolas(3). (1)Laboratoire de Bioluminescence, CNRS, 91190 Gif sur Yvette, (2)LERS, 58 rue de la Glacière, 75013 Paris, and (3)Laboratoire de Technologie, 105 Bd Raspail, 75006 Paris, France.

In the bioluminescent elytra of scale-worms (Annelids), two natural probes of luminescence and of a related fluorescence allow precise measures of the activity as well as acurate observation of the intracellular microsources through microscope and image intensifier. These photosomes, which are paracrystals of ER, are able to flash repeatedly. Under repetitive stimulation, an isolated elytrum emits a corresponding series of flashes which always begin by a period of facilitation because an increasing number of photosomes are set into activity. This recruitment is basically intracellular, photosomes being progressively coupled from the periphery to the center of each photogenic cell.

Coupling is a fast phenomenom, likely to be induced by the Ca++ influx of the action potentials spreading over the photogenic epithelium, since facilitation increases with stimulation frequency or with external calcium concentration. The coupled state of the photosomes is labile; its basic life span, of the order of a second, is lenghtened by reinforcement under sustained stimulation. A mathematical modelisation of the evolution of the photogenic charge (i.e.the photoprotein polynoïdin) and of the level of coupling fits with the experimental results and thus accreditates the notion of a transient coupling.

After fast freeze fixation-substitution, coupled photosomes and them only are surrounded by a network of intermediate ER which reaches the nearest plasma membrane and develops along it a long junctional saccule. Often symmetrical, often situated along gap junctions, such dyadic junctional complexes appear to realise the internal but temporary route for the excitation-bioluminescence coupling.

159 A NEW METHOD TO PRODUCE LARGE, CYTOSKELETON-FREE ERYTHROCYTE VESICLES.
Gábor Lelkes and Ildikó Fodor. National Institute of Haematology and Blood Transfusion, H-1502 Budapest, POB 44, Hungary

Vesiculation of intact erythrocytes can be induced by decreasing their intracellular pH by titrating an unbuffered red cell suspension with 0.15 M HCl at room temperature and then heating the erythrocytes to a critical temperature value which depends on the intracellular pH. A linear correlation could be established between the intracellular pH and the corresponding temperature values causing vesiculation. For example, at an intracellular pH 6 vesiculation begins at 45°C, but at pH 5.6 vesicle formation occurs at 37°C while at pH 5.2 it can be observed at 29°C. The vesicles are large (1-2 um in diameter) haemoglobin-containing and completely free of cytoskeletal proteins as detected by SDS-polyacrylamide gel electrophoresis. Freeze-fracture electron microscopy showed that, while Acridine orange caused a prompt and maximal rate of intramembrane particle aggregation in these vesicles, no low pH-induced aggregation could be detected when vesicle formation was induced at an intracellular pH 5.5 at 37°C. We propose that the vesiculation is due to a purely physicochemical molecular mechanism which affects the state and dimension of the cytoskeletal meshwork. Moreover, the possible role of an altered haemoglobin-membrane interaction in preventing low pH-induced intramembrane particle aggregation in the cytoskeleton-free vesicles is considered.

160 INFLUENCE OF RED AND FAR-RED IRRADIATION ON THE ULTRASTRUCTURAL LOCALIZATION OF ACETYLCHOLINESTERASE IN THE CELLS OF OAT COLEOPTILE.
A.Tretyn, K.Kwiatkowska. Copernicus Univ.,Inst.Biology, 87-100 Toruń, Poland

Acetylcholinesterase /AChE/ activity was found in 4-day-old oat seedling grown in the dark. Ultrastructural cytochemical investigations have revealed that the product of enzymatic reaction occurs only on the external side of the plasma membrane of the coleoptile cells. After 15 minutes of irradiation with red light AChE activity in the coleoptiles stopped - this was reflected by the absence of the reaction product. The inhibition effect of red on AChE activity was completly reversed by far-red. After 15 min. irradiation with red followed by 30 min. irradiation with far-red the product of AChE enzymatic activity reappered on the outer side of the plasma membrane of the oat coleoptile cells. As a result of far-red irradiation, the product of enzymatic reaction also appeared in the nuclear perichromatine spaces and in cellular vacuoles.

Considering the results of the present work, it is suggested that photoconversion of the phytochrome is associated with inhibition of AChE activity.

161 RESPONSE OF BIOLOGICAL MEMBRANES TO THE EFFECTS OF ENVIRONMENT.
Anvar Mirakhmedov (1),Miassar Djuraeva,(2),Komil Ochilov (3),Djakhangir Khamidov(1) Institute of Biochemistry of the Academy of Sciences of Uzbek SSR,700143,prospect M.Gorky 56,Tashkent,USSR.

The chemicals,which are widely used in agriculture for raising of crop yields, increase the contamination of environment and have an effect on metabolism of animal calls.The results of investigations on membrane-active properties of phosphoroorganic compounds (PhOC) are describedin present work.It is well-known,that the toxic effect of many xenobiotics is based on modification of structural and functional properties of biological membranes.It is established by us,that under in vitro conditions PhOC inhibit energy-dependent functions of mitochondria with the decrease in value of respiratory control as a result,and the stimulation of membrane permeability for cations causes the alterations in the activity of lipid peroxidation system.These data indicate the membranotropic properties of PhOC.Thus,xenobiotics induce the alterations in permeability of biological membranes with the following disturbance in activity of enzyme systems of membranes,that leads to alterations in cell metabolism.

162 THE ROLE OF LIPID PEROXIDATION IN CYTOCHROME P-450 DEGRADATION IN HEPATOCYTES AND LYMPHOCYTES AND STABILIZING EFFECTS OF ANTIOXIDANTS. Valerian Kagan (1), Kiril Novikov (2), Evgenia Bogdanova (2), Leonid Prilipko (2). (1) Institute of Physiology, Bulgarian Academy of Sciences, 1113 Sofia, and (2) M.V.Lomonosov Moscow State University, 117234 USSR

Incubation of primary culture of hepatocytes was accompanied by spontaneous degradation of cytochrome P-450 (P-450) and by a decrease of its monooxygenase activities. Accumulation of lipid peroxidation (LPO) products also occurred. Addition of LPO inducers (Fe^{2+}-ADP, NADPH)to the medium led to a drastic acceleration of both processes. Synthetic free radical scavengers (butylated hydroxytoluene, 3-hydroxybenzene, alpha-naphtol) prevented LPO activation and protected P-450 against degradation. Phenolic derivatives of 3,4-benzpyrene, formed as a result of its hydroxylation, also had an antioxidant and a protective effect on P-450. Similarly, incubation of human peripheral blood lymphocytes stimulated by PHA resulted in a rapid inhibition of arylhydrocarbon hydroxylase (AHH). In lymphocytes stimulated by PHA and then induced by 3-methyl cholantrene, the sharply increased AHH activity remained unchanged for a long period and no LPO products were accumulated. Exogenous LPO inducers did not either stimulate LPO and were ineffective on the AHH activity. This was due to the presence of 3-methyl cholantrene phenolic derivatives formed during lymphocytes induction. It is concluded that free redical scavengers could be used as P-450 stabilizers in cell cultures.

163 THERMAL PROPERTIES OF THE ERYTHROCYTE MEMBRANE AS DETECTED BY A SPIN LABELED STEARIC ACID. EVIDENCES FOR THE INVOLVEMENT OF SKELETAL PROTEINS

A.M.M. Di Stasi, T. Forte, M. Minetti, T.C. Petrucci. Lab. Biologia Cellulare, Istituto Superiore di Sanità, Viale Regina Elena 299 - 00161 Roma, Italy.

The involvement of skeletal proteins in thermal transitions of red blood cell (RBC) membrane, as detected by a stearic acid spin label (16-doxyl stearic acid) at 8°, 20°, 40°C has been investigated. Ca^{++}-treatment, extractions and rebinding of selective proteins and specific antibodies (Abs) have been used to modify membrane thermotropic properties. Ca^{++}-loading caused the lowering of the 40°C transition and the disappearance of the 8°C one. Electrophoretic pattern of Ca^{++}-treated membranes showed the disappearance of band 4.1. The 8°C transition was eliminated only by Anti-protein 4.1 Abs, while anti-spectrin Abs eliminated only the 40°C transition. The extraction of Band 6 (0.6 M KCl) did not modify membrane thermal properties, whereas the exhaustive extraction of spectrin and actin removed the 40°C transition. 1 M KCl extraction of inside-out vesicles, a procedure which extracts proteins 2.1 and 4.1, was able to eliminate the 8°C transition. Rebinding of purified protein 4.1 to the vesicles stripped of peripheral proteins restored the 8°C transition. These results suggest the involvement of protein 4.1 in the 8°C transition and the involvement of spectrin in the 40°C transition. Thermal properties of both purified and membrane bound spectrin as detected by circular dichroism and maleimide spin labeling studies showed a characteristic thermal unfolding of spectrin above 40°C. This thermotropic behavior of spectrin could be the process that, above 40°C, modifies the freedom of motion of the spin label. The interaction of skeletal proteins with the membrane seems to contribute significantly to the physico-chemical properties of the RBC membrane.

164 INVOLVEMENT OF PLASMA MEMBRANE PROTEINS IN CELL RESPONSE TO HYPERTHERMIA.

G. Arancia, G. Mariutti*, P. Trovalusci and W. Malorni. Department of Ultrastructures and Department of Physics*, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy.

In recent years many studies have been carried out on the cell response to hyperthermic treatment also in view of the increasing interest in the use of hyperthermia in cancer therapy. However, the mechanisms involved in cell sensitivity to hyperthermic treatments are still poorly understood. In particular little is known about the involvement of the plasma membrane in the hyperthermic cell killing and in the heat induced thermotolerance. In order to clarify the role of the membrane proteins in the cell response to hyperthermia, a freeze-fracture study has been performed on Chinese hamster fibroblasts V79. The quantitative analysis carried out with an interactive image analyzer on the intramembranous particles (IMPs) of both protoplasmic (PF) and external (EF) fracture faces of the plasma membrane, allowed to reveal an increase of their density and size after 1 hour of hyperthermic treatment at 43°C. In addition, if we roughly consider IMPs spherical in shape, it results that heated cells contain nearly twice as much particle volume per μm^2 of membrane as control cells do. Because it has been accepted that IMPs represent a visualization of membrane proteins, one can assume that under our experimental conditions, V79 cells respond to the heat shock by increasing the protein membrane content. Preliminary results obtained by scanning electron microscopy and immunocytochemistry seem to indicate that hyperthermia can also influence the arrangement of cytoskeletal components.

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165 CONTROVERSAL DATA TO THE HYPOTHESIS OF MEMBRANE PORE INDUCING ABILITY OF ANTIFUNGAL POLYENES

E. K. Novák/1/, Judit Zala/1/, I. Vincze/2/, M. Pesti/3/. /1/ Mycology Department, /2/ Biochemistry Department, National Institute of Hygiene, H-1966 Budapest POB 64; /3/ Research Station, Hungarian Seed Association, H-6601 Szentes, POB 41, Hungary

Finkelstein and Holz, /1973/, and de Kruijff and Demel /1974/ interpreted the ion leakage caused by antifungal polyenic macrolide antibiotics as: complexing with membrane sterol transmembrane pores are formed through which solutes of vital importance escape the cell passively. Our data when measuring ion fluxes with conductometry or 42-K and 24-Na tracers show divergency from the hypothesis. The ion leakage caused by the antifungal concentration of nystatin /Nys/ can be reduced by antimetabolites /iodoacetamid, azide/ in Candida albicans and by anaerobiosis in Rhodotorula rubra yeasts. Moreover Nys doesn't cause the penetration of Ni-II ions, as well as constitutively non permeating amino acides /arg, his, lys/. Spontaneous efflux of Ca ions from preloaded cells is not increased by Nys. The Nys caused ion leakage however, can promtly be increased only by constitutively metabolized sugar /glc./ but not by inductively metabolizable ones /xyl, sorbose/.

On the other hand the Nys caused ion leakage per se cann't be generalized for the group, as amphotericin B, flavofungin, pimaricin and candicidin cann't induce it in biologically relevant concentrations.

166 RELATIONSHIP BETWEEN MEMBRANE POTENTIAL CHANGES AND TRANSMITTER RELEASE IN RAT BRAIN SYNAPTOSOMES

Erzsébet Ligeti (1) and Vera Ádám-Vizi (2).(1) Department of Physiology and (2) 2nd Institute of Biochemistry of Semmelweis Medical University, Budapest, H-1444 POB 259

Membrane potential of isolated cortical synaptosomes was assessed on the basis of ^{86}Rb-distribution and correlated to the amount of acetylcholine released under identical conditions. The quantitative relationship of the two parameters depended on the way depolarization was achieved i.e. whether high K^+-concentration, ouabain or veratridine was used. A substantial depolarization (>15 mV) by K^+ was necessary to enhance acetylcholine release whereas already a slight depolarization (<5 mV) by the other two agents significantly increased the transmitter liberation. In calcium free medium the effect of high K^+-concentration was suppressed but ouabain and veratridine could still trigger the release process. Manipulation of the intracellular calcium stores either by the mitochondrial uncoupler ClCCP or by the calcium ionophore A23187 in the absence of external calcium resulted in acetyl choline release without any concomitant change in the membrane potential or the activity of the plasma membrane Na,K- ATPase enzyme. Mobilization of internal calcium stores as a consequence of increased intracellular Na^+-concentration could thus participate in the process of transmitter release in nerve endings.

167 INCREASE IN THE NUMBER OF LARGE INTRAMEMBRANE PARTICLES IN THE PRESYNAPTIC MEMBRANE DURING TRANSMISSION OF A SINGLE NERVE IMPULSE.

A. Parducz(1), L.M. Garcia-Segura (2), D. Müller (3), Y. Dunant (3). (1) Institute of Biophysics, Biological Research Center, Szeged, Hungary, (2) Institut d'Histologie et d'Embriologie,Geneve and Instituto Cajal, CSIC Velazquez 144 Madrid, Spain, (3)Département de Pharmacologie, Centre Médical Universitaire, 1211 Geneve 4, Switzerland

By using fast-freezing technique small pieces of Torpedo electric organ were cryofixed at 1 ms time intervals in a liquid medium at -190 °C before, during and after the passage of a single nerve impulse. The experiments were done in physiological conditions i.e. without 4-AP or any other drug able to potentiate transmitter release. The rate of cooling was high enough in the most superficial layers of the tissue to provide in freeze-fracture replicas of well-frozen material in the absence of any chemical fixation. We found that transmission of an impulse was accompanied by momentary occurrence of a population of large intramembrane particles in both the P and the E leaflets of the presynaptic membrane. The change was very brief, appearing soon after the stimulus artifact and lasted for 2-3 ms. This population of particles did not appear when stimulation was applied in a low-calcium medium, a condition where the nerve terminals were still depolarized by the action potential but did not release the neurotransmitter. The number of large pits denoting processes of endo- or exocytosis did not increase during the passage of the nerve impulse. From these and other observations, we conclude that this transient change of intramembrane particles is closely linked to the mechanism of acetylcholine release at the nerve-electroplaque junction.

168 CALCIUM CURRENT IN MOUSE EGGS MEASURED WITH THE PATCH AND WHOLE-CELL VOLTAGE CLAMP.

Antonio Peres. Dipartimento di Fisiologia e Biochimica Generali, Università di Milano, via Celoria 26, 20133 Milano, Italy.

The voltage-dependent, transient, calcium current present in mouse eggs (Okamoto et al., 1977) has been studied using the tight-seal, whole-cell, voltage-clamp and the patch-clamp techniques. Both techniques have several advantages over the conventional two-microelectrode voltage-clamp: whole-cell recording offers greatly improved signal to noise ratio, longer duration experiments and the possibility of introducing various substances inside the egg. Patch-clamp reveals single channel currents.

Both techniques are easily appliable to mouse eggs after enzymatic removal of the zona pellucida in an external medium containing 20 mM Ca^{2+}.

Different kinds of single channel currents are apparent in patch-clamp recordings. Of special interest are channels which have voltage- and time-dependent openings very similar to the macroscopic Ca^{2+} current. A detailed study of these channels is presently in progress and hopefully complete data will be presented at the Congress.

Okamoto, H., Takahashi, K. and Yamashita, N. (1977). Ionic currents through the membrane of the mammalian oocyte and their comparison with those in the Tunicate and Sea Urchin. J. Physiol. 267, 465-495.

169 HORMONAL IMPRINTING AND MEMBRANE POTENTIAL, THEIR COMMON ASPECTS

László Kőhidai /1/, Yoshinori Muto /2/, Yoshinori Nozawa /2/, György Csaba /1/
/1/ Dept. of Biology Semmelweis Univ. of Med. Budapest, Hungary
/2/ Gifu Univ.School of Med. Dept.of Biochem. Gifu, Japan

Single and double hormone-treated Tetrahymena GL Zeuten cells were examined as model objects of membrane alterations.
Binding of FITC labelled materials /insulin, TSH, FSH, and BSA/ and the changes of membrane potential were measured, by fluorescent techniques.
The different peptides possessing hormonal activity produce membrane depolarization and a special binding, according to the size of molecules and the repeats of treatments. The state of membrane - the down-regulation-effects - also influence the binding-profil of the membrane.
The membrane depolarizations are followed by marked, elevated hormone binding, while the repolarized membrane of the cells has an increased binding of labelled hormones.
This relation of membrane potential with hormonal imprinting or repeated treatments seems to be specific to hormone molecules; the non-hormone BSA has no effect in respect of neither the hormonal imprinting nor the membrane-potential alterations.

170 EFFECTS OF CHANGES IN SALINE IONS CONTENT AND OF MANNITOL ADDITION ON VOLUME AND ULTRASTRUCTURE OF CULTURED PC12 CELLS

E. Delpire, C. Duchêne, M. Cornet and R. Gilles
Laboratory of Animal Physiology, University of Liège, 22 Quai Van Beneden, 4020 Liège, BELGIUM.

Confluent cultures of rat pheochromocytoma cells (line PC12) submitted to anisosmotic conditions show important changes in cell ultrastructure. These are essentially located to the nucleus. In hypo-osmotic media, there is a marked decondensation of chromatin and the nucleolus is profoundly desorganized. Upon application of hyperosmotic shocks, the chromatin becomes more condensed and the nucleolus shows a more diffuse granularity.

Physiological studies show that application of anisosmotic conditions lead to changes in cell hydration as well as in osmolality, ionic strength and specific ions content of the intracellular fluid. The cell ultrastructure modifications can thus be related to one of these factors. To distinguish among them, we have studied the cell volume and ions intracellular content evolution in relation with changes in cell ultrastructure in different anisosmotic conditions induced by modifications in saline ions level and/or by mannitol addition. Our results demonstrate that the ultrastructural changes observed must be related to ions specific effects rather then to effects mediated through changes in cell hydration or intracellular fluid osmolality.

171 PHYSIOLOGICAL AND ULTRASTRUCTURAL EFFECTS OF OUABAIN IN RABBIT AND RAT RENAL SLICES RECOVERING AT 27°C FROM SWELLING AT 0.5°C

C. Duchêne, E. Delpire, M. Cornet and R. Gilles
Laboratory of Animal Physiology, University of Liège, 22 Quai Van Beneden, 4020 Liège, BELGIUM.

The present study deals with the effects of ouabain on the volume restoration process occurring in rabbit and rat kidney-cortex slices when recovering at 27°C from swelling at 0.5°C.

It is known from previous works that ouabain (1mM) completely blocks volume recovery in rabbit slices while that compound, even in much larger concentration (10mM), remains without significant effect on the process in rat slices. This has been taken as evidence to consider that the Na/K pump is playing an essential role in volume restoration, the lack of inhibition in rat slices being explained by the insensitivity of that tissue to ouabain (Cooke K.R., 1981).
Our present results show that in rabbit slices, ouabain does not only block volume restoration but also induces an intracellular accumulation of Na at a concentration higher than equilibrium with external medium. On the other hand, ouabain also induces changes in mitochondria size and electron density. These effects cannot be explained simply by considering inhibition of a Na/K pump by the cardiac glycoside. They will be discussed in terms of a possible effect of the drug on other pumping mechanisms involved in cell volume regulation of kidney cells.

Ref.: Cooke K.R., J. Physiol. Lond. 320, 319-332 (1981)

172 STRUCTURAL AND FUNCTIONAL ASPECTS OF CELL-CAM 105, AN EPITHELIAL CELL ADHESION MOLECULE.
Björn Öbrink (1), Per Odin (1), Anders Tingström (1), Magnus Hansson (1), Ingrid Blikstad (1) and Peter Svalander (2). Department of Medical and Physiological Chemistry (1) and Department of Anatomy (2), University of Uppsala, BMC, S-751 23 Uppsala, Sweden.

It is generally agreed that intercellular recognition and adhesion have important roles in the development and maintenance of tissue structure and function. In recent years the molecular dissection of intercellular junctions and the discovery of cell adhesion molecules (CAMs) have made the molecular mechanisms of cell adhesion phenomena discernible. Several CAMs have been discovered (1). In adult organisms CAMs occur selectively in various tissues, e.g. some CAMs are found preferentially in nervous tissues, while others are expressed mainly in epithelia.

Cell-CAM 105 is an epithelial CAM which was identified by immunological methods as being involved in cell-cell adhesion of rat hepatocytes in vitro (2). Chemical characterization of cell-CAM 105 purified from rat liver membranes has demonstrated that it consists of two structurally similar, highly N-glycosylated polypeptide chains of apparent molecular weights 105 000 and 110 000, respectively. Both chains can be phosphorylated on serine residues. Internal labelling of hepatocytes in culture has demonstrated that both chains are synthesized at approximately the same rate.

Pure cell-CAM 105 inhibits intercellular adhesion of hepatocytes, which indicates that the protein is directly involved in cell-cell binding. In order to study the binding mechanism cell-CAM 105 incorporated into liposomes has been utilized. Such liposomes bind specifically to hepatocytes and show a prominent calcium-independent self-aggregation. Thus, cell-CAM 105 seems to be able to bind to itself in a homophilic calcium-independent reaction.

Affinity-purified antibodies have been used in immunohistochemical studies of the cell surface localization of cell-CAM 105, both in liver sections and in cultured, primary hepatocytes. Both in the intact liver and in the cultured hepatocytes the glycoprotein is present on all faces of the cells. In the mature liver it is more concentrated to the bile canaliculi, whereas in cultured hepatocytes, lacking functionally mature bile canaliculi, the highest concentration is seen in all cell-cell contact areas of adjacent cells.

By immunochemical and immunohistochemical techniques cell-CAM 105 has been found also in other tissues, mainly mature, simple epithelia (3). The molecular size of cell-CAM 105 varies among different tissues, demonstrating that several molecular species exist. These techniques have also been used to monitor cell-CAM 105 in developing, regenerating, and malignant liver. In fetal liver no cell-CAM 105 is expressed before day 16 of the gestation (4), in regenerating liver a transient decrease of the concentration of cell-CAM 105 is observed (4) and in transplantable hepatocellular carcinomas it is either completely missing or drastically reduced in amount (5). These data indicate that cell-CAM 105 exerts its major function in terminally differentiated cells.

In rat preimplantation embryos cell-CAM 105 appears stage-specifically as determined by immunofluorescence microscopy. In morulae no cell-CAM 105 was detected, but on day 4 blastocysts it appears on the cell surface of the trophoblasts of the trophectoderm. At the time of implantation it disappears from the trophoblasts of the abembryonal pole, but not from the embryonal pole, of the blastocysts. The abembryonal pole represents the portion of the blastocysts that first attaches to and invades into the uterine mucosa.

In considerations of the physiological function(s) of cell-CAM 105 we want to emphasize three things: I) the occurrence of cell-CAM 105 primarily in terminally differentiated cells; II) the inverse correlation between occurrence of cell-CAM 105 and invasive potential of cells (hepatocellular carcinomas and implanting trophoblasts are invasive, whereas normal, mature hepatocytes and non-implanting trophoblasts are non-invasive); III) the looser organization and higher motility of cells having no or little cell-CAM 105 (fetal, regenerating and malignant hepatocytes are more loosely organized (4) and more motile than mature hepatocytes). Accordingly, we speculate that cell-CAM 105 confers properties on the cells that express it, that I) are responsible for a proper cellular and multicellular organization, II) make the cells staionary, respecting their territorial borders, III) prevent them from invading surrounding tissues. As mentioned above cell-CAM 105 has been found primarily in certain epithelia. However, preliminary data indicate that several molecular species occur. This observation suggests that similar, analogous molecules with similar functions may occur in other tissues as well.

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173 THE MOLECULAR BIOLOGY OF HUMAN FIBRONECTIN.
Raymond J. Owens and Francisco E. Baralle, Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, UK.

Fibronectin is a ubiquitous extracellular glycoprotein that is involved in a variety of cell contact processes. Reflecting its multiple roles, fibronectin shows a modular organisation comprising three different internal homology repeats (types I, II and III). These units are arranged in a highly conserved pattern which correlates with the location of specific binding sites along the length of the molecule. The application of molecular biology to the analysis of fibronectin structure has enabled a complete primary sequence of the protein to be deduced. The model of fibronectin structure based on plasma fibronectin has been verified for the cellular form of the protein. It has been shown that a number of different fibronectin mRNAs are produced by two processes of alternative splicing in the single fibronectin gene. One involves the deletion of an exon coding for a complete type III repeat (Extra Domain - ED), whilst the other involves the subdivision of an exon to give a non-homologous segment of variable length (type III Connecting Segment - IIICS). If all combinations of splicing are possible, then ten human fibronectin mRNAs may be generated by these events. Some of these fibronectin mRNAs account for subunit variants of the protein and show tissue-specific expression. The mechanism responsible for alternative splicing of the ED is currently being investigated.

The modular structure of the fibronectin protein appears to be reflected in the organisation of the gene. It is now known that the type III homology units of fibronectin are each encoded by two exons (with the exception of the alternatively spliced ED). To investigate the gene organisation of the type I and II repeats, λ genomic clones covering approximately the N-terminal third of the human fibronectin gene have been isolated. Sequences corresponding to the collagen-binding domain have been partially characterised. The results show that the type I and type II units are each coded for by single exons. These data lend general support to a gene fusion model for fibronectin evolution.

With the exception of the cell-binding site, which has been mapped to a tetrapeptide sequence (RGDS), very little is known about the exact structure of the other binding sites of fibronectin. Of these, the collagen-binding site is particularly important. The interaction beween fibronectin and collagen appears to be fundamental to the organisation of extracellular matrices and the behaviour of cells on these substrates. The collagen-binding domain of plasma fibronectin has been variously isolated as a 30-45 kD mol.wt and is characterised by the presence of the only type II internal repeats in the molecule in addition to a number of type I units. The unique occurrence of the type II units together with their high level of amino acid conservation implicates these sequences in collagen-binding. The availability of cDNA clones covering this region has enabled this to be tested directly. A series of overlapping cDNAs has been produced which encompasses the collagen-binding domain. These have been expressed as cro/β-galactosidase fusions in E.coli and the resulting hybrid proteins assayed for gelatin-binding activity. The results show that the actual collagen-binding site lies well inside the region defined by proteolytic cleavage of fibronectin, and appears to be localised to a sequence(s) within the type II homology repeats. Experiments are in progress to further define the binding site and to identify the residues critical for collagen binding.

174 FIBRONECTIN FRAGMENTS, PLASMINOGEN ACTIVATORS AND MALIGNANT TRANSFORMATION. Sergio Barlati, Giuseppina De Petro, Marina Colombi, Anna Adamoli, Lucia Rebessi. Istituto di Biologia, Università di Brescia, 25124, Italy.

The detection of Transformation Enhancing Factors (TEF) in in vitro and in vivo systems (1,2) indicates that correlations exist between the presence of TEF and the transformed state. The results obtained indicate that proteins involved in the fibrinolytic cascade and degradation products of extracellular matrix components may be responsible for the TEF activity evidenced in the culture medium of transformed cells (3,4,5) and in the plasma of cancer patients (6) and tumor-bearing mice (7). The demonstration that purified gelatin-binding fibronectin fragments (8,9), as well as t-PA (10) exert TEF activity, together with the detection of these proteins in crude or partially purified TEF-active fractions, strongly supports this possibility. It has been also shown that proteins involved in blood coagulation (11), have associated Transformation Inhibiting (TI) activity. These findings indicate that a balance between activation of the enzymes and proteins involved in fibrinolysis and coagulation might be an important parameter for favoring the expression of a transformed or a normal phenotype respectively. A scheme which summarizes this hypothesis is reported in Fig. 1. In this model are taken into account both normal and transformed cells, and an extracellular compartment, in which proteins of connective tissues and body fluids may interact with the cellular system in a series of reciprocal interactions. The activity of proteolytic enzymes may be exerted on Extracellular matrix (EXM) proteins as well as on connective tissues and/or body fluids protein components. EXM degradation products (EXMdp) and degradation products of some plasma proteins (i.e. fibronectin) may all contribute to the generation of TEF activities. EXM degradation products

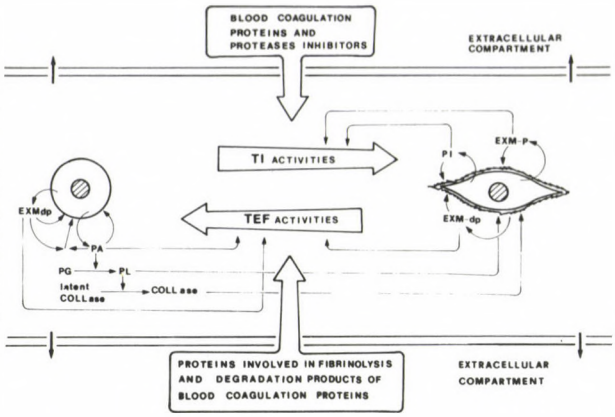

Fig 1.
Model proposed for the correlations occurring between: proteins released by normal cells (Extracellular Matrix Proteins: EXM-P, proteases Inhibitors: PI), transformed cells (Plasminogen Activators: PA, EXM degradation Products: EXMdp), blood plasma proteins (FN, Fibrinogen, Thrombin etc.), enzymes activated by PAs (Plasminogen: PG, Plasmin: PL, Collagenases: Collase), extracellular components (connective tissues) and TEF and TI activities.

may act by competing with the intact proteins for a specific binding site on the cells or on other matrix components and thus prevent the formation of an organized EXM. Moreover, the activation of the fibrinolytic cascade in body fluids may generate local conditions which favor the expression of the transformed phenotype. All these activities could act in establishing the microenvironment which favors cell proliferation and transformation once the activation of oncogenes has been attained.

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175 FIBRONECTIN-BINDING CELLULAR PROTEINS IN NORMAL AND MALIGNANT CELLS: THE ROLE OF FIBRONECTIN IN CELL ATTACHMENT. Ismo Virtanen, Veli-Pekka Lehto and Tapio Vartio. Department of Pathology, University of Helsinki, Haartmaninkatu 3, SF-00290 Helsinki, Finland.

We have studied parameters related to the cell adhesion function of fibronectin and to the defective organization of the pericellular matrix in transformed and malignant cells.

When normal human fibroblasts are plated after trypsinization in the presence of monensin, a secretion inhibitor, the cells both adhere and spread but do not form stress-fibres or focal adhesion sites. This adhesion process cannot be inhibited by the arg-gly-asp-ser peptide, exhibiting the cell attachment promoting activity of fibronectin (Fn). However, exposure of the spreading cells to cellular fibronectin rapidly brings about both stress-fibres and focal adhesions. Studies using iodinated Fn from normal or malignant cells in a direct overlay assay of electrophoretically separated cellular proteins transferred onto nitrocellulose sheets showed that there are two Fn-binding proteins in plasma membranes of normal but not of malignant cells, MW 140kD and 170kD. These polypeptides could be revealed also by using a 120kD fragment of Fn, containing the cell binding site. The malignant cells studied showed a comparable 170kD Fn-binding polypeptide in the overlay assay of whole cell lysates but not of plasma membrane preparations. Moreover, surface-labeling studies indicated that most of the malignant cells apparently express the 140kD protein which appears to lack Fn-binding property.

The results suggest that Fn has a direct role in the organization of the microfilament system, probably mediated by specific binding-proteins. At least two membrane proteins may function in this event. On the other hand, Fn produced by malignant cells appears to interact normally with these proteins. Thus, the defect in fibronectin-matrix assembly in malignant cells appears to be due to alterations in the surface-expression or binding-properties of the Fn-binding proteins rather than to a defect in fibronectin itself.

176 MECHANISMS OF EMBRYONIC CELL MIGRATION IN THE VERTEBRATES.

Jean Paul Thiery(1), Jean Loup Duband(1), Sylvie Rocher(1) and Kenneth M. Yamada(2).
(1)Institut d'Embryologie CNRS, 94736 Nogent-sur-Marne, Cedex FRANCE and NCI, NIH Bethesda Md. 20205 USA.

The extracellular matrix (ECM) associated with epithelial tissue basement membranes and with the mesenchyme plays a key role in tissue remodelling and cell migration. Fibronectin (FN), appears prior to or at the blastula stage in the vertebrate embryo. In amphibians, FN assembles as a fibrillar network on the roof of the blastocoelic cavity preceding mesodermal cell migration. The latter do not migrate in regions of the roof where the ectoderm has been inverted, providing an ECM-free substrate. Microinjection of monovalent antibodies to FN in the blastocoelic cavity arrests gastrulation. The same effect is obtained after injection of a synthetic decapeptide containing the cell binding site sequence of FN. Neural crest cells migrate along or between FN-rich basement membranes. In birds, pathways of migration have been traced using antibodies to FN and a monoclonal antibody which identifies crest cells. In most cases, crest cells maintain a high density in transient narrow pathways. Directionality of migration is in part given by population pressure and does not seem to require other guidance mechanisms such as chemotaxis or haptotaxis. Crest cells adhere preferentially to FN both in vitro and in vivo. Antibodies to FN, and the cell binding peptide inhibit migration on FN substrates in culture and microinjection blocks cephalic crest cell migration in embryos..Using a combined in vivo and in vitro approach, we have analyzed the immunofluorescent localization and function of a 140kilodaltons glycoprotein complex implicated in cell adhesion to fibronectin (FN).This putative fibronectin receptor complex (FN-receptor) was detectable in almost all tissues derived from each of the three primary germ layers. It was present in both mesenchymal and epithelial cells, and was particularly enriched at sites close to concentrations of FN, e.g. at the basal surfaces of epithelial cells. It was also present on neural crest cells. In vitro the putative receptor was diffusely organized on highly locomotory neural crest cells and somitic fibroblasts. Both motile cell types also displayed relatively low numbers of focal contacts and microfilament bundles and limited amounts of localized vinculin,α-actinin, and endogenous FN. In contrast, the FN-receptor in stationary embryonic cells, i.e. somitic cells after long-term culture or ectodermal cells, existed in characteristic linear patterns generally codistributed withα-actinin and fibers of endogenous FN. Anti-FN-receptor antibodies inhibited the adhesion to FN of motile embryonic cells, but not of stationary fibroblasts. However, these same antibodies adsorbed to substrata readily mediated adhesion and spreading of cells, but were much less effective for cell migration. Our results demonstrate a widespread occurrence in vivo of the putative FN-receptor, with high concentrations near FN. Embryonic cell migration was associated with a diffuse organization of this putative receptor on the cell surface in presumably labile adhesions, whereas stationary cells were anchored to the substratum at specific sites linked to the cytoskeleton near local concentrations of FN-receptor. These data provide both circumstantial and direct evidence for the importance of FN in directed cell migration. The distribution and function of the cell adhesion molecules (CAMs) were also established during early embryogenesis particularly at the time of neural crest cell appearance migration and arrest. The two primary CAMs: N-CAM and L-CAM appear on all cells on the blastoderm. During gastrulation and neural crest cell migration both primary CAMs disappear from the surface of emigrating cells. N-CAM is expressed de novo at the time of crest aggregation both in vivo and in vitro. Ng-CAM, a secondary CAM involved in neuron-glia and neuron-adhesion is expressed only on post-mitotic neurons and immature Schwann cells. The spatially and temporarily controlled expression of cell-cell and cell-ECM adhesion molecules is a major factor in the patterning of embryos.

177 ROLE OF LAMININ, FIBRONECTIN AND COLLAGEN IN SKELETAL MUSCLE DEVELOPMENT. Klaus von der Mark, Uwe Kühl, Mücella Öcalan and Gundula Risse. Max-Planck-Institut für Biochemie, D-8033 Martinsried, FRG.

The extracellular matrix of embryonic skeletal muscle consists in principle of a reticular connective tissue matrix containing types I, III and V collagen and fibronectin, and a basement membrane matrix ensheathing each individual myofiber. The muscle basement membrane contains type IV collagen, laminin, nidogen, and heparan sulfate proteoglycan. All these components are synthesized and secreted by muscle connective tissue cells as well as mononucleated prefusion myoblasts; type IV collagen and laminin are deposited on the myotube surface, assembling into a basal lamina.

Laminin and fibronectin have contrary effects on development and differentiation of skeletal muscle: myoblasts adhere rapidly to laminin and type IV collagen, while muscle fibroblasts have a preference for fibronectin and type I collagen. These differential affinities may play a role in the adhesion of fusion-capable myoblasts to growing myotubes, thus selecting for myogenic and against fibrogenic cells.

Furthermore, laminin but not fibronectin induces spreading and elongation of myoblasts in culture and stimulates proliferation in a dose-dependent manner when used as solid culture substrate. Fibronectin also stimulates proliferation, but to a lower extent; it causes modulation to fibroblast-like cells and delays fusion.

These observations suggest specific interactions between myoblasts and laminin. Western blotting of muscle cell membranes with radiolabelled laminin demonstrated a major laminin-binding protein of M_r 68,000 in addition to 4 other components of M_r 160,000, 120,000, 90,000 and 47,000. The 68kD protein (LB68) was isolated by affinity chromatography on laminin sepharose and characterized by peptide mapping and amino acid analysis. After insertion into liposomes it binds laminin, but not fibronectin or collagen. It is presumably involved in one or several laminin-myoblast interactions and thus may play an important role in muscle development.

178 LOCALIZATION OF EXTRACELLULAR MATRIX (ECM) PROTEINS DURING INTESTINAL MORPHOGENESIS AND IN CO-CULTURES OF EPITHELIAL CELLS WITH FIBROBLASTS.

P.M. SIMON-ASSMANN*, M. KEDINGER*, R. TIMPL** and K. HAFFEN*

*INSERM U.61, Strasbourg, France. **Max-Planck Institut für Biochimie, Martinsried bei München, RFA.

The aim of this study was to analyze the distribution of ECM components as a function of intestinal morphogenesis and differentiation. For this purpose, the immunocytochemical localisation of basal membrane (bm : type IV collagen, laminin, nidogen), and interstitial matrix (im : type III procollagen, fibronectin) components, has been analyzed 1) in situ, at various stages of development ranging from 12 days of gestation to the adult, 2) in vitro, in co-cultures of 14 days fetal intestinal endodermal cells (embryonic epithelial cells) seeded on a confluent monolayer of fetal intestinal or skin fibroblasts.

In the developing intestine, bm components were present at the epithelial/mesenchymal interface whereas im proteins were found throughout the whole mesenchyme, at the earliest stage studied. As soon as 15 days of gestation, before villus formation, modifications in the distribution and intensity of all antigens within the mesenchyme parallel the orientation and segregation of mesenchymal cells in the region which surrounds the bm. Furthermore, during villus outgrowth, a transient disappearance of procollagen III at the top of the protruding villus core is obvious and when crypts develop, the staining of the bm proteins became more pronounced at the base of the villi. In the adult organ, no major changes along the crypt-villus axis are found in relation to epithelial proliferation and differentiation. In vitro, in the culture conditions in which the epithelial cells displayed a morphological and enzymatic differentiation, assessed by their polarization, the presence of apical brush borders and the synthesis of digestive enzymes, the ECM proteins studied were synthesized and found at their specific localisation (at the epithelial/fibroblastic interface and/or within the fibroblastic cell layer).

These data suggest that epithelial/mesenchymal interactions, whose role in intestinal development has already been demonstrated, could be directed via ECM components.

179 Estrogen regulates and v-ras^H Transfection constitutively induces Interactions of MCF-7 Breast Carcinoma Cells with Basement Membrane. Adriana Albini*, Jeannette O. Graf, Gregory T. Kitten, Hynda K. Kleinman, George R. Martin, Andre' Veillette and Marc E. Lippman. NIH/NIDR/NCI, Bethesda, MD

MCF-7 cells are a line of human breast cancer cells derived from a pleural metastasis of an adenocarcinoma. Estrogen stimulates the growth of these cells in culture and is an absolute requirement for for tumor growth when the cells are injected into nude mice. A subline of MCF-7 cells transfected with with the v-ras^H oncogene is able to form tumors even without estrogen supplements. In our studies, the ability of these cells to cross a reconstituted basement membrane was used as a test for their invasiveness. We found that the v-ras^H transformed cells readily crossed the reconstituted basement membrane. The MCF-7 cells were not invasive when grown without estrogen. With estrogen (17β-estradiol) they showed a dose-dependent increase in invasiveness. Other differences were noted with these cells which may be related to the differences in invasiveness. MCF-7 cells deprived of estrogen were found to attach poorly to laminin and were unable to migrate toward laminin. In contrast, MCF-7 cells exposed to estrogen as well as v-ras^H transfected MCF-7 cells were found to attach to laminin and they also migrated towards laminin. Since such interactions are likely to be mediated via specific cell surface receptors, we measured the binding of ^{125}I laminin to the cells. These studies showed that the number of laminin receptors on the MCF-7 cells was increased 2-3 fold by estrogen or by v-ras^H transfection. In summary, these studies indicate that the invasive phenotype of the MCF-7 cell type is controlled by estrogen and is expressed in the absence of estrogen in the MCF-7 cells transfected with v-ras^H probably through different mechanisms. The level of receptors for laminin is increased under these conditions and may mediate some of these responses.

*Permanent Address: Istituto Nazionale per la Ricerca sul Cancro, 16 132 Genova (Italy)

180 CHONDROCYTES FROM TRANSIENT CARTILAGE ONLY DIFFERENTIATE TO HYPERTROPHIC, TYPE X COLLAGEN-PRODUCING CHONDROCYTES IN ANCHORAGE INDEPENDENT CULTURE CONDITION.

P.Castagnola, F.Descalzi-Cancedda, G.Moro and R.Cancedda - IST, Istituto Nazionale per la Ricerca sul Cancro, Viale Benedetto XV, Genoa, Italy.

Type X collagen is selectively synthesized by hypertrophic chondrocytes in regions characterized by a remodeling of the cartilage matrix leading to the replacement of the cartilage with bone. The relationship between the differentiation of endochondral chondrocytes and the synthesis of type X collagen was investigated in a culture system starting from chondrocytes obtained from 29-31 stage chick embryo tibiae. The cells at the beginning of the culture were small and synthesized type II but not type X collagen (stage I chondrocytes). When they were grown on agarose coated dishes, condition that does not allow cell adhesion, they continued their differentiation to hypertrophic chondrocytes synthesizing type X collagen (stage II chondrocytes). In anchorage dependent growth condition stage I chondrocytes dedifferentiated and switched from the synthesis of type II to the synthesis of type I collagen. The synthesis of the collagens was followed both analysing the labeled proteins made by the cells and measuring the levels of the collagen mRNAs by northern blot. As probes we used cDNA clones for the types I and II collagen received from other groups and a cDNA clone for the type X collagen obtained in our laboratory. After several passages in condition of anchorage dependent growth, dedifferentiated chondrocytes were transfered on agarose coated dishes. In this new condition they reexpressed the differentiated phenotype and continued their differentiation to endochondral hypertrophic chondrocytes synthesizing type X collagen. Hypertrophic chondrocytes, obtained growing the cells on agarose, when plated on anchorage permissive dishes maintained the differentiated phenotype and continued the synthesis of type X collagen.

181 ORIENTATION PATTERN OF PERICELLULAR MATRIX COMPONENTS IN EMBRYONIC CARTILAGE

László Módis, Zoltán Szekanecz. Institute of Anatomy, Histology and Embryology, University Medical School, H-4012 Debrecen, Hungary.

It is generally believed that the extracellular matrix (ECM) of the hyaline cartilage is an amorphous system. Electron microscopic histochemical and polarization optical investigations, however, revealed on oriented microstructure of the ECM.

We studied the spatial orientation of proteoglycans (PGs), glycosaminoglycans (GAGs) and collagen in embryonic chicken and human hyaline cartilages of different ages. Using electron microscopic histochemical reactions for PGs and polarization microscopic analysis of topo-optical reactions specific for GAGs and collagen, the following orientation pattern of these matrix components could be described. /i/One group of PGs is attached to the cell membrane. The long axes of these PGs are ordered perpendicularly to the tangent of the membrane. /ii/ The chondroitin sulfate side chains of these PGs are arranged parallel to the membrane. This ordered PG and GAG structure precedes the formation of collagen fibrils./iii/ The subsequently formed collagen fibrils assume the same orientation pattern as described for GAGs. This is probably due to the ionic interactions of these components. /iv/ As the differentiation of collagen structure proceeds, two other localizations of PGs can be distinguished:numerous PGs are bound periodically and perpendicularly to the collagen fibrils, while other PGs remain randomly distributed in the interfibrillarly space. /v/ Cell shape strongly influences the orientation pattern. Spherical cells are surrounded by circularly arranged collagen fibrils and GAG molecules, and longitudinally oriented collagen+GAG structure develops in the vicinity of flattened cells.

182 CELL-CELL ADHESION MEDIATED BY CELL-CAM 105 IN RAT HEPATOCYTES.

Anders Tingström and Björn Öbrink, Department of Medical and Physiological Chemistry, University of Uppsala, Uppsala, Sweden.

Cell-CAM 105 is a 105 000 dalton cell surface glycoprotein which has been isolated from adult rat liver. The glycoprotein is involved in reaggregation of isolated adult rat hepatocytes and it has been purified by immunoaffinity chromatography on antibodies that inhibit this process. In the present investigation we show that cell-CAM 105 can be incorporated into phosphatidylcholine liposomes. These vesicles aggregate spontaneously during preparation and dissociate if anti-cell-CAM 105 Fab is added. The aggregation is calcium-independent since it is unaffected by EGTA-treatment. The liposomes also bind specifically to isolated rat hepatocytes. To understand the physiological role of this adhesive mechanism we have studied the localization of cell-CAM 105 in primary hepatocyte cultures using immunofluorescence microscopy. In unpermeabilised hepatocyte monolayers cell-CAM 105 was seen on free cell surfaces, i.e. areas where the cells are not in contact with each other, and only a faint irregular staining was seen at cell contacts. However, if the cells were pereabilised with Triton X-100 after fixation, or if they were treated with a calcium-free buffer before fixation, which made them to separate, an intense staining was seen at all sides where the cells were in close contact. In the permeabilised cells we also noticed a clear disappearance of the staining of the free surfaces. These results indicate that cell-CAM 105 may be organized in different ways at free cell surfaces and in the cell contact areas. The adhesive properties of liposome-incorporated cell-CAM 105 and the presence of the protein in cell contact areas suggest that cell-CAM 105 directly mediates hepatocyte-hepatocyte adhesion via a calcium-independent homophilic binding mechanism.

183 STRUCTURE, TISSUE DISTRIBUTION AND DEVELOPMENTAL BIOLOGY OF CELL-CAM 105.
Per Odin and Björn Übrink, Department of Medical and Physiological Chemistry, University of Uppsala, Uppsala, Sweden.

Cell-CAM 105 is an integral membrane protein that has been shown to be involved in cell-cell adhesion of isolated rat hepatocytes. It has been purified to homogeneity from rat liver membranes. Biochemical characterization has shown that the liver protein consists of two glycosylated peptide chains with apparent molecular weights of 105 000 and 110 000, respectively. Peptide mapping suggested that the two chains are nearly identical. Amino acid and carbohydrate analyses demonstrated that the carbohydrate accounts for 33% of the mass of the glycoprotein. The following saccharides were found: mannose, galactose, fucose, sialic acid and N-acetylglucosamine. No N-acetylgalactosamine was found, indicating the presence of N-linked oligosaccharides only. Proteolytic cleavage with papain and subtilisin together with analysis of characteristic autodegradation products indicate that cell-CAM 105 contains several well-defined domains. By radioimmunoassay, immunoblotting and immunohistochemical techniques the occurence of cell-CAM 105 was investigated in various tissues. The glycoprotein was found primarily in simple epithelia. However, at least three different size classes with different migration patterns in SDS-PAGE were found. The immunochemical and immunohistochemical techniques were also used in analyses of cell-CAM 105 in developing and regenerating liver. In fetal liver no cell-CAM 105 is expressed before day 16 of the gestation. At birth (day 21) the concentration of cell-CAM 105 is only 1/3 of that in mature liver, which is not reached until 3 weeks postnatal. In regenerating liver the concentration of cell-CAM 105 in the plasma membranes of the hepatocytes drops to 1/3 of the normal 2-3 days after partial hepatectomy. Around day 10-15, when the liver growth ceases, the amount of cell-CAM 105 is back to normal again. These results indicate that cell-CAM 105 exerts its major physiological function in terminally differentiated cells.

184 THE PRESENCE AND TENTATIVE FUNCTION OF THE CELL ADHESION MOLECULE CELL-CAM 105 IN INTESTINAL EPITHELIAL CELLS.
Magnus Hansson, Per Odin, Björn Übrink. Department of Medical and Physiological Chemistry, Uppsala University, Box 575, 751 23 Uppsala, Sweden.

Cell-CAM 105 is an integral cell surface membrane glycoprotein that has been shown to be involved in rat hepatocyte intercellular adhesion. Using monospecific rabbit anti-cell-CAM 105 antibodies in indirect immunofluorescence studies, it has been possible to demonstrate the presence of cell-CAM 105 in various rat tissues, predominantly in simple epithelia. In small intestine the immunofluorescence staining is concentrated to the microvilli of the enterocyte apical surface. This is in accordance with the pattern observed in liver sections, where the bile canaliculi are heavily stained, and only a faint fluorescence can be seen around the basolateral surface of the cells. This staining pattern may either be due to a higher concentration of cell-CAM 105 in the microvillar membrane as compared to the basolateral plasma membrane, or may simply reflect the large amount of membranes that are packed in the microvillar region. Cell-CAM 105 has been purified from rat hepatocyte membranes by immunoaffinity chromatography, and small intestinal cell-CAM 105 has been similarly purified from isolated enterocyte microvilli. In SDS-PAGE, liver cell-CAM 105 shows an app M_r of 90 kD whereas small intestinal cell-CAM 105 has an app M_r of about 115 kD. Under reducing conditions the app M_r are 105 kD (liver) or 90 kD (small intestine), respectively. Possibly, small intestinal cell-CAM 105 is associated with another molecule, that is dissociated by reduction. These differences might indicate a different function of cell-CAM 105 in enterocytes as compared to hepatocytes, or that the function of cell-CAM 105 is regulated in different ways. The concentration of cell-CAM 105 to the microvillar regions of the cells has led us to speculate that cell-CAM 105 might be involved in microvillar organization and mechanochemical regulation of microvillar motility. Essential features of this model are that cell-CAM 105 both binds adjacent microvilli together and interacts with the core-bundle of microfilaments.

185 INTERACTIONS OF TYPE I COLLAGEN WITH THE CELL MEMBRANE.
Marino Bortolussi (1), Ornella Selmin (1), Giorgio Bressan (2), Dino Volpin (2). (1) Institute of Human Anatomy and (2) Institute of Histology and Embryology, University of Padua, Italy.

The role played by the extracellular matrix in cell adhesion, proliferation and differentiation is well known. We have studied the binding of 125-I-labelled human type I collagen to fibroblasts and the effects of monoclonal antibodies (MAbs) raised against fibroblasts on cell adhesion. The binding of 125-I-collagen to monolayers of human dermal fibroblasts and bovine fetal ligamentum nuchae fibroblasts (110 days) reached the equilibrium in 2-3 h at 26°C and was a saturable process with a Kd of 10^{-8}M. The binding of 125-I-collagen to monolayers was markedly increased if the ligand was preheated at 50°C for 30 min before incubation. The characteristics of the binding of 125-I-collagen to purified membranes of the fibroblasts were similar to those obtained with the monolayers. The dissociation of 125-I-collagen bound to the membranes was significantly increased by the addition of excess unlabelled collagen I, but it was only scarcely affected by an equimolar concentration of fibronectin. Affinity chromatography of detergent extracts of membranes of human fibroblasts on a column of agarose-collagen I resulted in the enrichment of a 33 Kd species. We have obtained a set of MAbs directed against surface antigens of human fibroblasts as evidenced by immunofluorescence and radioimmunobinding. Preliminary results indicate that some of these MAbs inhibit markedly the adhesion of such fibroblasts to collagen I but not to fibronectin. The results of these studies suggest the presence of membrane receptors specific for collagen I on human and bovine fibroblasts.

186 FIBRONECTIN AND RELATIONSHIPS OF THE FIRST EMBRYONIC TISSUES IN THE COW BLASTOCYST. Véronique Richoux(1,2), Jacques-E. Fléchon(1), Thierry Darribère(2), Jean-Claude Boucaut(2). (1) Unité de Biologie du Développement, Département de Physiologie animale, I.N.R.A. 78350 Jouy-en-Josas, and (2) Laboratoire de Biologie expérimentale,U.A. C.N.R.S. 1135, Université R. Descartes, 45 rue des Saints-Pères, 75270 Paris cédex 06, France.

Early cow blastocysts (day 7) are surrounded by a layer of trophectoderm cells and contain an inner cell mass. The next step (day 8 on) is the differentiation of endoderm cells from the inner cell mass and their migration around the blastocoele. Electron microscopy shows that endoderm cells are flat mesenchyme-like cells making only focal contacts with the inner aspect of the trophectoderm epithelium, underlined with a basal lamina-like extracellular matrix. Using antibodies against purified bovine fibronectin, this component of the matrix was localized by indirect cytochemical techniques in a continuous layer on the basal face of the trophectoderm epithelium. We suggest that endoderm cells migrate on this extracellular matrix and especially use fibronectin as a substratum. In elongating blastocysts (day 12), the trophectoderm over the inner cell mass disappeared and the outer layer of the latter was differentiated into embryonic ectoderm. Before the polar trophectoderm (Rauber layer) was lost, fibronectin was found between it and the inner cell mass. Just before the Rauber layer was disrupted, the extracellular matrix between the trophectoderm and the inner cell mass appeared to be discontinuous at the ultrastructural level. When the embryonic disc became prominent outside the trophectoderm, there was no more fibronectin on its surface. The degeneration of the Rauber layer cells might be related to the disaggregation of the underlying basal lamina.

187 SUBCELLULAR LAMININ DETECTION IN ADULT MALE GLANDULAR RAT ANTERIOR PITUITARY CELLS. Evelyne Vila-Porcile (1) and Claude Tougard (2). (1) Laboratoire d'Histologie, Faculté de Médecine Pitié-Salpêtrière, Paris and (2) Groupe de Neuroendocrinologie Cellulaire et Moléculaire, Collège de France, Paris, France.

Laminin, a major component of basement membranes, has been detected on the basal laminae and also within some glandular cells of the pituitary (Tougard et al., In Vitro, 1985,21, 57-61). Since it has been previously found that intracellular detection of laminin was depending upon technical procedures, optimal conditions were adjusted, using an immunoperoxidase pre-embedding method.

Under such conditions, all the pituitary cell categories, identified either on morphological or on immunocytochemical criteria, appeared to contain laminin. Each of these cell types displayed differences in the intensity of their reaction and in the subcellular distribution of laminin.

In every cell category, the rough endoplasmic reticulum was labeled at a more or less extent: heavily in lactotropes, gonadotropes and thyrotropes, weakly in the other cell types. The labeling of Golgi saccules was discrete in all the cells. Most of the stored secretory granules were immunoreactive in gonadotropes, thyrotropes and sometimes corticotropes but not in somatotropes and lactotropes. However, granules on exocytosis process were found labeled whatever the cell type. These extruding granules might represent a way for laminin exportation towards basal laminae. Immunoreactive vesicles, located in the Golgi zone and at the cell periphery, were particularly numerous in lactotropes, but fewer in other cell categories, thus raising the question of their possible role in laminin exportation or in an eventual reuptake of components from extracellular matrix.

These observations suggest that laminin could be synthesized, transported and exported by all of the glandular pituitary cells. Whether the differences observed in the subcellular distribution of laminin are linked to the rate of cell activity or to the existence of various secretory pathways depending on the pituitary cell type remains to be elucidated.

188 DIFFERENTIAL ADHESIVE CAPACITIES OF CHICK EMBRYO FIBROBLASTS (CEF) ON FIBRONECTIN AND LAMININ SUBSTRATA.
CODOGNO P., DOYENNETTE M.-A. AND AUBERY M. Unité INSERM 180, CNRS UAC 81, 45-rue des Saints-Pères 75006 Paris, France.

For several years, many studies have focused on the glycoproteins implicated in cell attachment to the extracellular matrix: fibronectin, laminin, chondronectin and vitronectin mainly. Their localization and association with collagen types are well documented too. Mesenchymal derived cells use fibronectin whereas epithelial and endothelial cells use laminin to adhere to their respective extracellular matrix. Furthermore, these attachment glycoproteins interact with integral components of the plasma membrane. Such putative receptors have been recently described for laminin and fibronectin respectively. Herein, we show that after a mild trypsin treatment CEF are able to adhere, in a very similar manner, on fibronectin and laminin substrata, respectively. The specificity of the CEF adhesion toward the protein used (i.e. fibronectin and laminin) was shown using antobodies against laminin and fibronectin, and soluble forms of fibronectin and laminin during the adhesion assays. Pretreating the cells with cycloheximide (inhibitor of the protein biosynthesis) and monensin (a monovalent ionophore which inhibits the transport of glycoproteins from the Golgi apparatus to the cell surface) prevents the adhesion of CEF on laminin but not on fibronectin coated dishes. Furthermore, preincubation of the cells at low temperature (4°C, 21°C) before the adhesion assay performed at 37°C delays the CEF atachment to laminin substratum only.

189 LOCALIZATION OF FIBRONECTIN IN CULTURED HUMAN SKIN FIBROBLASTS AS VISUALIZED BY DIFFERENT IMMUNOCYTOCHEMICAL TECHNIQUES

Bärbel Harrach, Detlef Veltel and Horst Robenek
Lehrstuhl für Medizinische Cytobiologie der Universität Münster, D-4400 Münster, Domagkstr. 3, Federal Republic of Germany

To contribute to the visualization of components of the extracellular matrix we have studied the localization and distribution of fibronectin in cultured human skin fibroblasts. We used pre-embedding peroxidase, post-embedding gold labelling and surface replication techniques at the electron microscopical level. Immunofluorescence at the light microscopical level was carried out for comparison. After treatment of the cells with an antibody against fibronectin and labelling with protein A-gold complexes, the surface replicas reveal long strands of gold particles at the cell surface.
Visualization of fibronectin in the interior of the cell by the immunoperoxidase method requires pretreatment with detergents such as saponin or Triton X. Both substances strongly affect the structure of cells and organelles. In fibroblasts the saponin concentration of o.o5% allows not only penetration of the antibody into the cell, but also visualization of the reaction product in the rough endoplasmic reticulum and the Golgi apparatus.
These results are confirmed by the post-embedding immunogold labelling using a protein A-gold procedure. Fixation in 1% glutaraldehyde and embedding in Lowicryl K4M sufficiently preserves the structure of fibroblasts to localize fibronectin in the rough endoplasmic reticulum and the Golgi apparatus.
Supported by SFB 310

190 FIBRONECTIN REAPPEARANCE AND CELL ADHESION. Joëlle Botti and Michèle Aubery

Unité INSERM 180, CNRS UAC 81, UER Biomédicale des Saints-Pères, 45 rue des Saints-Pères 75006 Paris, France.

The cell surface fibronectin plays a crucial role in the growth, morphology and adhesive properties of animal cells, and particularly in fibroblast adhesion and spreading onto the substratum. After release by chemical treatment or loose by after transformation of cell surface fibronectin, cells became rounded and poorly adhesive.

We have previously demonstrated that fibroblasts from chick embryos aged of 8 days readhered to the substratum of culture more rapidly after their mild treatment with trypsin than those of 16 days. We investigated the reappearance of membrane fibronectin at the surface of trypsinized embryo cells.

The time course of the reappearance of membrane fibronectin at the surface of embryo cells, after their detachment from the substratum by trypsin treatment, was correlated to the time course of their capacity to readhere to the substratum. Both membrane fibronectin reappearance and cell readhesion to the substratum were reduced by monensin, an inhibitor of the protein export from the golgi apparatus to the plasma membrane. The membrane fibronectin reappeared earlier in 8 day (30 min) than in 16 day (60 min)as showed by chemical and immunological methods: time course of membrane fibronectin after metabolic labelling of embryo cells and extraction of labelled fibronectin by urea treatment, polyacrylamide gel electrophoresis analyses of labelled membrane fibronectin after immunoprecipitation at various times of cell readhesion.
These observations were confirmed by indirect immunofluorescence staining of embryo cells readhering to the substratum at various times of readhesion.

191 THE INTERACTION OF THE INTEGRAL PLASMA MEMBRANE PROTEIN 5'-NUCLEOTIDASE AND LAMININ IS REQUIRED FOR FIBROBLASTS SPREADING.

CODOGNO P.[1],DOYENNETTE M.-A.[1],AUBERY M.[1],DIECKHOFF J.[2],LIETZKE R.[2] and MANNHERZ H.G.[2] (1)Unité INSERM 180, CNRS UAC 81, 45-rue des Saints-Pères 75006 Paris, France. (2)Institut für Anatomie und Zellbiologie, Philipps-Universität, 3550 Marburg, W.Germany.

Fibroblasts are known to adhere on various substrata (e.g. fibronectin, laminin, lectins and glycosyltransferases). Recently, cell surface receptors for extracellular molecules (i.e. fibronectin and laminin) have been identified. Nevertheless, the cell spreading process involves integral plasma membrane glycoproteins related to the cytoskeleton too. In the present work, we show that 5'-nucleotidase, an ectoenzyme concentrated in the plasma membrane, which hydrolyses AMP into adenosine and inorganic phosphate, and recently shown to interact with filamentous (F-) actin is involved in the spreading process of chick embryo fibroblasts (CEF) after their initial attachment to a laminin substratum. Moreover, monoclonal antibodies which do not or slightly inhibit (less than 25 %) the AMPase activity of 5'-nucleotidase lead to an important impairment of CEF spreading, whereas monoclonal antibodies which strongly inhibit (more than 95 %) the AMPase activity exhibit only a very slight effect on the CEF spreading on laminin substratum. In the same manner, the inhibition of the AMPase activity with competitive inhibitors (, -methylene-ADP or , -imido-ATP) did not perturb the spreading of CEF on laminin substratum. However, it should be noted that antibodies against 5'-nucleotidase do not impair CEF spreading on a fibronectin substratum.

192 HUMAN ERYTHROLEUKEMIA CELLS (K562) ADHERE SPECIFICALLY ON FIBRONECTIN-COATED SUBSTRATA. Tapio Vartio and Ismo Virtanen. Departments of Virology and Pathology, University of Helsinki, Haartmaninkatu 3, SF-00290 Helsinki, Finland.

Human K562 erythroleukemia cells typically grow in suspension as rounded cells. When cultured in serum-free medium K562 cells rapidly adhered on substratum coated with fibronectin (Fn) but not on that coated with laminin. Many of the cells underwent also a rapid spreading process, resulting in flattened, often angular cells. The spreading process could be quantitatively inhibited with the peptide, arg-gly-asp-ser, corresponding to the cell attachment site of fibronectin. The spreading process also brought about polarization of the actomyosin system, as visualized with NBD-phallacidin. In suspended cells, actin-microfilaments are peripherally located throughout the cells whereas in adhering K562 cells actin becomes rapidly polarized into peripheral lamellae and microspike-like structures.

Direct iodinated Fn-overlay assay of electrophoretically separated cellular proteins transferred onto nitrocellulose sheets indicated that there is a major 110kD Fn-binding polypeptide in K562 cells. The adhesion of K562 cells on Fn responded differently to various differentiation-inducing drugs. Exposure to the tumor promoter,TPA, enhanced the adhesion capacity whereas exposure to hemin completely inhibited the adhesion process. After the hemin-induced differentiation, the major Fn-binding polypeptide could not anymore be revealed in the overlay assay.

The present results show that K562 erythroleukemia cells show a highly specific ability to adhere and spread on Fn, which process is apparently mediated by a specific receptor-protein in these cells. Thus, it may be that also *in vivo* adhesion of erythroid cells on Fn may participate in regulation of normal or pathologic erythropoiesis.

193 REGULATION AND RECONSTITUTION OF INTERPLATELET RECOGNITION DURING AGGREGATION. Galila Agam, Irit Deutsch, Avinoam Livne. Department of Biology, Ben-Gurion University of the Negev, Beer-Sheva (Israel)

Fixed platelets bearing covalently bound fibrinogen were previously shown to participate passively in release-related aggregation. Purified thrombospondin (TSP) was proven to be the released compound which specifically and selectively recognizes the affixed fibrinogen. The present study demonstrates that the phenomenon of passive participation is also obtained with fixed platelets bearing covalently bound TSP. Moreover, a full resolution and reconstitution of interplatelet recognition during aggregation was obtained with two different systems: A) Fixed platelets bearing affixed fibrinogen were caused to aggregate when stirred and supplemented with soluble TSP. B) Fixed platelets bearing fibrinogen and fixed platelets bearing TSP, each uncapable of undergoing aggregation, aggregated when combined and stirred. It is concluded that these two non-membrane glycoproteins - fibrinogen and TSP - play a major role in the molecular mechanism of interplatelet recognition during aggregation.

194 EFFECT OF EXOGENOUS HUMAN PLASMA FIBRONECTIN ON PLATELET AGGREGATION. Tereza Rusanescu, Maya Simionescu, Institute of Cellular Biology and Pathology, Bucharest - 79691, Romania

We used an in vitro system to study the effect of Fn on (a) the inhibition of ADP-induced platelet aggregation and, (b) desaggregation of ADP-aggregated platelets. Fn was isolated from human normal plasma by affinity chromatography on gelatin-Sepharose 4B and purified on DEAE cellulose. Human platelet rich plasma (PRP) was obtained from blood collected on acid-citrate-dextrose, centrifuged at 165xg for 10 min at room temperature. Platelet aggregation by ADP was monitored by spectrophotometry, by light microscopy and controlled by electron microscopy. Fn (50 to 300 µg) added to PRP followed by ADP (1-5 µM), inhibited platelet aggregation in a dose dependent manner. These results were confirmed by light and electron microscopy. Control experiments in which Fn was previously absorbed onto gelatin-Sepharose 4B or gelatin or procedures in which Fn was replaced by the vehicle buffer (50 mM Tris buffer) had no effect on ADP-induced platelet aggregation. Also, inhibition was partially abolished (~40-60%) when exogenous Fn was previously incubated with IgG anti-plasma Fn. To further test the possible desaggregatory effect of plasma Fn, the latter was added in vitro to ADP-aggregated platelets and its effect was monitored in time. The desaggregating effect of Fn was time, and concentration dependent: it required higher concentration and longer time than in the case of inhibition of aggregation. These experiments show that excess Fn can act both in preventing aggregation and to some extent has a desaggregating effect. The mechanisms of this dual action of Fn are probably different. By specific binding of Fn to fibrinogen/fibrin it may interfere directly in ADP aggregation. The desaggregating effect of Fn may be explained by its ability to accelerate plasmin formation which in turn is fibrinolytic. (Supported by Ministry of Education, Romania, and by NIH Grant HL-26343).

195 TIME KINETICS AND ULTRASTRUCTURAL DISTRIBUTION OF FIBRONECTIN PRODUCTION IN SYNOVIAL MEMBRANE CULTURE. T.Neumark/1/, Katalin Merétey/1/, J.Török/1/, S.Neumüller/2/, B.Magyar/1/, A.Dunky/2/, G.Partsch/2/, R.Eberl/2/. /1/ National Institute of Rheumatology and Physiotherapy, H-1027 Budapest, Frankel Leó u.17-19. and /2/ Ludwig Boltzmann Institute of Rheumatology and Balneology, A-1107 Wien--Oberlaa, Kurbadstrasse 10.

Synovial tissue fragments from patients with rheumatoid arthritis /RA/ and other inflammatory arthritides were put into tissue culture flasks/Nunc/ for primary explantation. The supernatant was changed twice a week and tested for fibronectin, IgG and IgM levels. The growth rate and the metabolic activity were followed. The glycoprotein-rich coat on the surface of fibroblasts in subcultures was also studied by electron microscopy with the help of ruthenium red, with labelling of carbohydrates by horseradish peroxidase attached to Concavalin A and by immunoelectron microscopy with antifibronectin antibody. The fibronectin within the membrane was localized in patches, which was distinct from the continous presence of the sialic acid in the surface coat. These results show that can survive rather long time within a fragment producing immunoglobulins and that the fibronectin is a good marker for the control of the metabolic activity of synovial fibroblasts. The ageing of the cultures is resulted in a longer period between confluensies and a normal or increased activity in fibronectin production.

196 THE ROLE OF DIVALENT CATIONS IN CELL-CELL AND CELL-SUBSTRATUM ADHESION

Proto Pippia(1), Gloria Tilloca(1), Maria Antonietta Meloni(1), Augusto Cogoli(2), Giorgio Ivaldi(1). (1) Institute of General Physiology and Biological Chemistry, Sassari University, I-07100 Sassari, Via Muroni 25, Italy, and (2) Laboratorium für Biochemie, ETH-Zentrum, CH-8092 Zürich, Switzerland

Cell adhesion is fundamental to many diverse biological processes like growth, differentiation and contact inhibition. The study of the role of divalent cations in cellular adhesion is complicated by heterogeneity of both the experimental methods used and of the cellular systems investigated. Therefore in this research we adapted to our experimental conditions a method that allows us to measure at the same time cell-cell and cell-substratum adhesion. This method consists of determining the percent of single cells labeled with tritiated L-leucine adhering to a confluent monolayer of unlabeled cells or to polystyrene at different incubation times. The research has been performed on a strain of rat fibroblasts (FG/2). The results obtained from our experiments on cell-cell adhesion are in contrast with the observations described by others in previous reports. In fact the cell-cell adhesion kinetics obtained with different Ca^{++} and Mg^{++} concentrations show that both ions are required for optimum adhesion. Increasing the concentration - from 1.25 to 2.5 mM of Ca^{++} and from 0.8 to 1.6 mM of Mg^{++} - of one of them does not compensate the absence of the other. On the contrary, the loss of adhesion capacity to the substratum - observed when using a medium deprived either of Ca^{++} or of Mg^{++} - may be compensated by doubling either the concentration of Ca^{++} and leaving out Mg^{++}, or viceversa. This confirms (i) the hyphotesis that the divalent cations provide an electrostatic bridge between the cells and the substratum, (ii) that in cell-substratum adhesion this cations are required for cytoskeleton function rathen than as adhesion bonds per se, and (iii) that molecular base of cell-cell and cell-substratum adhesion is different.

197 STRUCTURE AND FUNCTION OF TOPOSOMES IN SEA URCHIN DEVELOPMENT. Valeria Matranga*, Melchiorre Cervello*, Hans Noll°. *Istituto di Biologia dello Sviluppo C.N.R., Via Archirafi 20, 90123 Palermo, Italy, and °Department of Biochemistry, Northwestern University, Evanston, IL 60201, USA.

A morphogenetic microtiter assay that measures the reaggregation into developing embryos of dissociated cells from sea urchin blastulae (Matranga et al., in press) was used to purify the cell surface component responsible for cell adhesion. We found that all cell adhesion activity was associated with a 22S genus-specific glycoprotein complex consisting initially of six 160-kDa subunits that are processed proteolitically as development proceeds. Non cytolitic removal of the 22S particle from the surface with either 2.5% butanol or trypsin renders dissociated cells reaggregation incompetent, and addition restores reaggregation and development. Polyclonal antibodies against the 22S complex prevent reaggregation in a genus-specific manner, while monoclonal antibodies stain cell surface structures in a pattern consistent with a code that specify the position of a cell in the embryo by a unique combination of subunits in its 22S particles (Noll et al., 1985, Proc.Natl.Acad.Sci. USA 82, 8062). Our results are consistent with a two-site model in which reaggregation results when 14S trimers on different cells associate by a Ca^{2+}-dependent homophilic interaction to form 22S hexamers. This association site is blocked by antibodies and Con A whereas the site binding the complex to the cell is sensitive to trypsin and 2.5% butanol. The existence of similar glycoprotein complexes in Drosophila (Wilcox et al., 1984, EMBO J. 3, 2307) and amphibian embryos (Slack, 1984, J. Embryol.Exp.Morphol. 80, 289) suggests that these glycoprotein complexes are a general class of organelles, the toposomes, that in the embryo mediate cell adhesion and express positional information.

198 PARENCHYMAL/STROMAL RELATIONS, AS MEASURED HISTOPHOTOMETRICALLY ON SIRIUS RED F3BA STAINED SECTIONS, IN DEVELOPMENT, REGENERATION AND CHOLESTATIC FIBROSIS IN RAT AND HUMAN LIVER

J. James, K.S. Bosch, D. Aronson, N. Lygidakis and J. van Gool. Laboratory of Histology and Cell Biology, Laboratory for Experimental Medicine and Department of Surgery, Academic Medical Center, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

Under normal conditions, the amount of extracellular matrix in the liver, and in particular the collagenous fiber scaffold, is kept at a more or less constant ratio to the parenchymal cell mass. This constant parenchyma/stroma ratio is generally thought to remain constant during physiological growth and also following partial hepatectomy, although only limited direct data are available in this respect. By means of histophotometric analysis of Sirius Red F3BA stained sections of rat livers at different stages of postnatal development, and also following partial hepatectomy, direct data about the volume density of collagen have been accumulated in this respect, which have been compared with the events during the development of cholestatic fibrosis in rats following ligation of the ductus choledochus and in patients suffering from a tumour in the hepatic duct. Results of these investigations will be presented, also taking into account some aspects of the actual production of collagen at the cellular level.

199 ULTRASTRUCTURAL EVIDENCE OF AN EASILY REMOVABLE ACCUMULATION OF NEGATIVE SURFACE CHARGES IN ASSOCIATION WITH THE INTERCELLULAR CLEFT IN CONTINUOUS MICROVESSELS.

Bengt R. Johansson (1), Sven Stener (1), Börje Haraldsson (2). Departments of Anatomy (1) and Physiology (2), University of Göteborg, P.O. Box 33031, S-400 33 Göteborg, Sweden.

Observations were made on artificially perfused rat hearts and hindquarters after bolus injections with cationized ferritin (CF) and ionic lanthanum (La^{+++}). CF injected within 2 min after start of perfusion decorated the luminal surface of heart and muscle microvessels as small lumps (cf Simionescu & al J Cell Biol 90:605 1981) including a prominent deposit adjacent to and regularly covering the intercellular cleft. During post-labelling perfusion the CF was redistributed into fewer and larger aggregates that gradually became detached from the cell surface. Re-injection of CF did not restitute the original decoration pattern. La^{+++} particularly intensely stained the intercellular cleft throughout both its length and width, and extended as an electron dense protrusion into the vessel lumen when injected early after blood flow was replaced with artificial perfusion. Given 1 h after preperfusion La^{+++} labelled the cleft proper but did not engage the luminal orifice of the cleft. Prefixation with glutaraldehyde seemed to cause a collapse of the La^{+++}-binding substance at the cleft opening. Moreover, prefixation and prolonged perfusion decreased the width of the La^{+++} deposits within the cleft leaving a central non-La^{+++}-labelled electron translucent zone. In conclusion our findings indicate that in vivo there is an accumulation of an anionic substance that is removed by artificial perfusion with a balanced salt solution containing albumin in association with the interendothelial cleft in continuous exchange vessels. Provided that the intercellular pathway is ascribed a significant role in the permeation of macromolecules it is plausible that such a substance should reduce the transit of negatively charged plasma proteins. This is corroborated by determinations of permeability parameters: after 2 h perfusions with serum-containing perfusate the ^{125}I-albumin clearance is about three times lower than after perfusate containing equivalent amounts of albumin only whereas hydraulic conductivity is unchanged.

200 MACULA DENSA CELLS AND INTERCELLULAR REACTIONS TO DIABETES AND GLUCOSE INFUSION.

Ruth Rasch and Palle Holck. Department of Cell Biology, Institute of Anatomy, University of Aarhus, DK-8000 Aarhus C, Denmark.

In the kidney the macula densa, which is part of the juxtaglomerular apparatus, is located between the distal tubule and the lacis cells. The region is considered to play a role in the tubuloglomerular feed-back. However, the mechanism for the transmission of the signal through the macula densa is not entirely understood. In normal animals this region has intercellular spaces that are much larger than the intercellular spaces in the rest of the distal tubule.

The size of intercellular spaces has been measured in 5 streptozotocin diabetic animals with blood glucose levels of about 17 mmol/l after 50 days duration of diabetes and in 5 age matched control animals. In 2 additional experiments in each 5 normal animals, the intercellular space in the macula densa region was measured, 10 and 1 min after a glucose infusion resulting in blood glucose levels of about 22 mmol/l. At the termination of the experiments a perfusion fixation was performed. 40 serial sections were made of 3 randomly selected and orientated epon embedded tissue blocks. From each block 3 total juxtaglomerular apparatus were followed on the serial sections. From each juxtaglomerular apparatus 3 sections were randomly selected and 1-3 electron micrographs were taken from each section at a magnification of 20,000 x. Applying a point count technique the relative area of the intercellular space was calculated as % of the whole macula densa region.

In the normal animals the intercellular space in the macula densa region comprised 8.67% $\pm$ 4.4 (SD) but in the diabetic animals it was significantly lower 1.49% $\pm$ 1.4. In the animals given glucose infusions the intercellular space was 1.05% and 0.57% in the 10 and 1 min experiments, respectively. The present study has shown that the macula densa cells which change configuration in diabetes show similar changes after rapid rise in blood glucose, however, the significance of these morphological changes in the operation of the tubuloglomerular feed-back system remains to be learned.

201 FUNCTION OF ERYTHROCYTE CELL MEMBRANE IN IMMUNE REACTIONS.

Vladimir T. Vasilenko. Institute of Transplantology and Artificial Organs, USSR Ministry of Health,]23436, Moscow, Pehotnaya 2/3, USSR.

It has been establised for the first time by morphological, immunological, immunofluorescent, radiometric and radioautographic methods in our experiments that the recipient's erythrocytes can adsorb in vivo and in vitro some of antigens of a transplantated organ and so that they can transport these antigens to the immunogenic organs, where antigenic information is received by macrophages, lymphocytes and reticular cells.

The erythrocytes with this mechanism participate not only in the inductive phase of immune reaction but also serve as a mediator in the antigen neutralization reactions on the next step of immune response. Furthermore, the adsorption of antigenic material on the erythrocyte's surface prevents the simultaneous entrance of this material into the immunogenic organs. Thus, the erythrocytes serve as a perculiar buffer system, which regulates the intensity of immune response.

Probably, described phenomenon plays an important role in the initiation of antibody genesis after the hole organ transplantation. The lack of direct connection between graft's and recipient's lymphoid systems doesn't influence on the development of the immune reactions, due to the erythrocytes, which transfer to the recipient's immunogenic organs enough amount of antigenic material necessary to carry out the initial phase of immunogenesis.

202 MONOCLONAL ANTIBODIES TO THE EXTRACELLULAR MATRIX OF BOVINE ENDOTHELIAL CELLS.

P. Anne Underwood, Frances A. Bennett. CSIRO Division of Molecular Biology, P.O. Box 184, North Ryde, NSW 2113, Australia.

BALB/c mice received intraperitoneal injections of extracellular matrix (ECM) material prepared from bovine corneal endothelial cells in culture. Spleens of immune mice were used to prepare hybridomas secreting antibodies reactive with ECM. The panel of hybridomas so obtained exhibited a varied range of specificities for individual components of the ECM. Antibodies were obtained with specificities for fibronectin, heparan sulphate, elastin and type IV collagen. Western blotting, immunoprecipitation and treatment of matrix with specific enzymes identified a range of other specificities for proteins ranging in molecular wt from 31K to 230K. Details of these will be given in the presentation. The panel of monoclonal antibodies is to be used to investigate the interactions of a variety of cell types with the extracellular matrix.

203 MICRO-INJECTION OF PROBES AGAINST EXTRACELLULAR MATRIX IN EMBRYOS OF PATELLA VULGATA SPECIFICALLY INTERFERES WITH NORMAL DEVELOPMENT.

Wiel M. Kühtreiber, Cees A.M. van Dongen and Jo A.M. van den Biggelaar. State University of Utrecht, Department of Experimental Zoology, Padualaan 8, Utrecht, The Netherlands.

The determination of the stem cell of the mesodermal cell line in embryos of the equally cleaving molusc Patella vulgata is a clear example of the significance of cell position and of cellular interaction for development. All four macromeres at the vegetal pole of a 32-cell stage embryo have the capacity to form mesoderm. However, only the macromere that contacts the micromeres at the opposite animal pole and that establishes a central position within the embryo will be induced to develop mesoderm. During this process the radial symmetry of the embryo is shifted into a bilateral one. The mechanism which accounts for the transfer of the inductive signal to the central macromere is not known. Electron microscopical investigations show that an extracellular matrix (ECM) is present between the central macromere and the inducing micromeres shortly before and during the interaction phase. This ECM can be labeled with the lectins (Succinyl-) ConA, LCH-B and PEA. Micro-injections of picoliter quantities of these lectins (20 µg/ml in o.1 M KCl) into the blastocoelic cavity at the 16- or early 32-cell stage lead to radially symmetrical embryos in which all four macromeres behave in the same way. Apparently, induction of the mesodermal cell line fails to occur in these embryos. The same result can be achieved by the micro-injection of hyaluronidase (1 mg/ml in 0.1 M KCl). Control embryos that are injected with similar or higher amounts of 0.1 M KCl continue to develop normal. It is concluded that the ECM probably plays a role in the induction process, either directly by supplying the inducing signal itself, or indirectly as a means for cell guidance and/or anchorage of the interacting cells.

204 THE THREE-DIMENSIONAL ORGANIZATION OF THE COCK COMB EXTRACELLULAR MATRIX.

A. J. Cidadão, K. L. David-Ferreira and J. F. David-Ferreira. Department of Cell Biology, Gulbenkian Institute of Science, Oeiras, Portugal

The three-dimensional architecture of the cock comb extracellular matrix (ECM), was studied using a replica method developed in our laboratory (A. J. Cidadão and J. F. David-Ferreira, J. Microsc., 1986, in press). It consists of TEM observation of rotary-shadowed platinum-carbon replicas obtained from critical-point dried resinless sections of polyethylene glycol-embedded specimens. Ultrathin sections of Epon-embedded material were also studied. Glutaraldehyde-osmium fixation was used, and in some cases the cationic dyes ruthenium hexammine trichloride (Hunziker et al., J. Ultrastruc. Res., 81, 1, 1982) and Alcian blue (Scott et al., Histochemie, 5, 221, 1965) were added to improve proteoglycan preservation.

The observation of stereo-pairs from replicas allowed a good visualization of the ECM three-dimensional organization. Collagen fibrils, elastic fibers, and the proteoglycan network, were clearly visualized. Collagen transverse striation, as well as its subfibrillar organization, were observed. The amorphous and microfibrillar components of elastic fibers were also visualized. Granules decorating collagen, and filaments linking collagen/elastic fibers, were evident. The technique was particularly useful to demonstrate, in large zones, the continuity between ECM elements. Differences in ECM organization with the various fixation procedures were analyzed. Results demonstrate the complementarity between replicas and ultrathin sections in the study of ECM.

205 BIOCHEMICAL AND IMMUNOLOGICAL STUDIES OF THE HETEROGENEITY OF PROTEOHEPARAN SULFATES IN HUMAN LIVER PARENCHYMAL CELLS.

Uwe Schönrock, Kurt von Figura, Physiologisch-Chemisches Institut, Universität Münster, Waldeyerstr. 15, D-4400 Münster, FRG

The heterogeneity of proteoheparan sulfates in human liver parenchymal cells was investigated by biochemical and immunological means. Radiolabelled proteoheparan sulfates were isolated from the secretion of a human hepatoma cell line (Hep/G2), and by sequential extraction of the cell layer with 0.35 M NaCl, 2 M NaCl, and 2 M NaCl + 1 % Triton X-100 in 50 mM Tris, pH 6.8, in the presence of protease inhibitors. The fractions, designated as Medium, 0.35 M NaCl, 2 M NaCl, and Trition X-100 were purified on DEAE-Trisacryl, eluting with 0.45 M NaCl. Striking differences in the affinity to Octylsepharose were observed, showing that only 30 % of the radiolabelled proteoheparan sulfate from the Medium fraction had affinity to Octylsepharose, whereas over 91 % of the radiolabelled proteoheparan sulfate from the Triton X-100 fractions bound to the column. Gelfiltration on Sepharose Cl-6B showed differences in the elution profiles of the Medium and Triton X-100 fraction. The Medium fraction eluted with a K_{av} of 0.38, whereas material from the Triton X-100 fraction eluted with a K_{av} of 0.34 and with the void volume. After immunoprecipitation with antibodies raised against human liver proteoheparan sulfate and following SDS gelelectrophoresis, two populations with an apparent molecular weight of 65 000-175 000 and 205 000-410 000 were observed in the Medium fraction, while in the Triton fraction two species with an apparent molecular weight of 92 000-115 000 and 225 000-330 000 were observed. Heparitinase degradation of the two populations from the Medium fraction gave two protein cores with apparent molecular weights of 105 000 and 40 000, respectively. The molecular weight of the polysaccharide chains of the large and small proteoheparan sulfates was 16 300 and 18 700, respectively. These results point to a marked heterogeneity of proteoheparan sulfates synthesized in Hep/G2 cells.

206 RECEPTORS TO LECTIN FROM DOLICHUS BIFLORUS AT THE SYNAPTIC BASAL LAMINA OF THE NEUROMUSCULAR JUNCTION.

Mª Josep Bellmunt, Joan Ribera, Josep E. Esquerda, Mª Antònia Poca and Joan X. Comella. Fac. Medicine. Estudi General de Lleida, Anselm Clavé-18, E-25007 Lleida. S P A I N.

The binding of the agglutinin from Dolichus biflorus (DBA) to synaptic portion of muscle basal lamina, was studied with immunohistochemical methods. In the rat muscle, lectin binding is specifically detected in neuromuscular junction. Long term denervated end-plates, failed to bind DBA. DBA receptor appear later than AChR or AChE, during development of neuromuscular junctions; it is first visualized in 3 day old newborn rats. We have detected polymorphic differences in DBA binding among different species; synaptic binding occurs well in mammalians and reptilians but not in birds and fishes. The fact that DBA receptors appear later during development and their phylogenetic polymorphism, suggest us that their functional significance is not releated to synaptic transmission and it could be implicated in postnatalstabilization of neuromuscular synapses. A neuromuscular synapse enriched fraction was obtained from mice and extracted to obtain their extracellular matrix componets; when submitted to SDS-PAGE analysis, two low molecular species, that bind to DBA are found. These molecular componets are absent in extracellular matrix obtained from extrasynaptic regions of the muscle.

207 PROTEOGLICANS OF EPIDERMAL CELL IN PLANARIAN WOUND:AN ULTRASTRUCTURAL HISTOCHEMICAL ANALYSIS. Rita Pascolini, Daniela Vagnetti, Simonetta Tei. Institute of Comparative Anatomy, University of Perugia, Via A. Pascoli, 06100 Perugia, Italy

Planarian wound provides a convenient source of migrating epidermal cells well suited for motility studies. In order to determine how various proteoglicans (PG) modulate cell-matrix adhesive interactions, as a preliminary step, we examined their distribution by ultrastructural histochemistry using the following cationic dyes : ruthenium red, Alcian blue, acridine orange and safranine O. Specific identification was accomplished by digestion of material with chondroitinase ABC and nitrous acid (NA). Cationic dyes revealed chondroitinase ABC and NA resistent membrane plaques and exhibited affinity for two structural components of extracellular matrix (ECM) which differed by 1)overall size,2)localization within the matrix,3)sensibility to chondroitinase ABC and NA. NA susceptible and chondroitinase ABC resistent component of ECM indicates that an N-sulfated PG, likely heparan sulfate PG, is present mainly at the level of basal lamina.

208 INTRACELLULAR MEMBRANE TRAFFIC

Professor Graham Warren, Department of Biochemistry, University of Dundee, Medical Sciences Institute, DUNDEE DD1 4HN, Scotland, UK

The vesicle is the fundamental unit of membrane traffic. It is formed from planar membranes with the aid of a cytoplasmic coat and only those macromolecules destined for transport are taken up into it. Once the vesicle has pinched off the coat is removed and the uncoated vesicle then recognises and fuses specifically with the next compartment on the transport pathway. Since the half-life of the vesicle is only a matter of minutes the process is extremely rapid yet the in-built selectivity ensures that the composition of each of the participating compartments is not compromised. From what is already known about the steps on the endocytic and exocytic pathways scores of proteins must be involved, yet only a few have been characterised. The approaches taken by us and others, and the results obtained so far, will be discussed.

209 **MECHANISM OF PROTEIN IMPORT INTO MITOCHONDRIA**

G. Hawlitschek, H. Köhler, D. Nicholson, R. Pfaller, N. Pfanner, R. Stuart and **W. Neupert.** Institut für Physiologische Chemie, University of Munich, Goethestr. 33, 8000 München 2, FRG

The process of import of proteins into mitochondria can be subdivided into several steps. We will focus on two of these steps, namely i) specific recognition of precursors by receptors on the surface of the mitochondrion; and ii) insertion into, and translocation across mitochondrial membranes.

ad i) Determination of receptor sites and of parameters of receptor binding will be reported for an outer membrane protein (porin); an intermembrane space protein (cytochrome c); and for an inner membrane protein (ADP/ATP carrier). In the case of the first two proteins, precursor forms could be prepared from the isolated mature forms; thus, chemical amounts of precursors (ligands) are available for receptor studies.

ad ii) Transport of proteins into the inner membrane or into the matrix requires an energized inner membrane. This appears to be for a step following receptor binding. A study of the import of the ADP/ATP carrier has revealed that its translocation from the receptor-bound state into the inner membrane can be driven by a potassium diffusion potential. Further analysis identified the electrical membrane potential $\Delta\Psi$ (and not $\Delta\mu_{H^+}$) as the relevant energy form. Implications of these findings on the mechanism of membrane translocation will be discussed. Experiments on the translocation of apocytochrome **c** (the precursor to holocytochrome c) across the outer membrane will be described. This process does not require a membrane potential and appears to differ from the transmembrane transfer of all other precursors studied so far. Models for the molecular mechanisms of translocation of a number of proteins into various mitochondrial subcompartments will be presented.

210 SIGNAL RECOGNITION IN INTRACELLULAR PROTEIN TRANSPORT

T.A. Rapoport (1), M. Wiedmann (1), T.V. Kurzchalia (1), A. Huth (1), A.S. Girshovich (2), E.S. Bochkareva (2), H. Bielka (1). (1) Central institute for Molecular Biology of the Academy of Sciences of GDR, 1115 Berlin-Buch, Robert-Rössle Str.10, GDR, and (2) Institute of Protein Research, Academy of Sciences of USSR, 142 292 Poustchino, Moscow Region, USSR.

Hydrophobic signal sequences direct the translocation of nascent secretory and many membrane proteins across the endoplasmic reticulum membrane. Initiation of this process involves the signal recognition particle (SRP) which consists of six different polypeptide chains and a 7S RNA (1) (for review see ref.2).

Using a cell-free transcription-translation system we have shown that the signal sequence of carp preproinsulin is absolutely required for the functions of SRP. If the signal peptide is deleted from the corresponding gene, there are abolished both the translational arrest exerted by SRP in the absence of microsomal membranes, and the translocation across the endoplasmic reticulum membrane (3).

In order to provide more direct evidence for an interaction between the signal sequence and SRP, a new method of affinity labelling has been worked out. N^{ε}-p-azidobenzoyl-lysyl-tRNA was synthesized and shown to be accepted by the protein synthesizing machinery. Cell-free synthesized polypeptides contained the ε-modified lysine residues. Preprolactin was then used as a model since it contains two lysine residues just in front of the hydrophobic core of the signal peptide. An arrested complex was produced by synchronized translation of preprolactin mRNA in the presence of SRP. Crosslinking was induced by irradiation. A new band in a SDS-gel of a molecular weight of 60 KD was found. This product was immunoprecipitable by affinity purified antibodies to the 54 KD- polypeptide of SRP and thus represents its crosslinking with the arrested fragment of preprolactin (about 6 - 8 KD). These data provide direct evidence for an interaction of the hydrophobic signal sequence with a protein component of SRP (4).

In most cases, signal peptides are located at the N-terminus of an exported protein and are cleaved off after membrane transfer. We have shown that the signal sequence of carp preproinsulin remains functional with dog pancreatic SRP even if engineered to an internal location (5). The data are consistent with a loop model of protein translocation (6).

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211 DISSECTING THE GOLGI COMPLEX WITH ANTIBODIES. Hubert Reggio

Laboratoire de Biologie de la Differenciation Cellulaire; Faculté des Sciences de Marseille-Luminy,case 901 13288 Marseille cedex 9. FRANCE.

Antibodies were raised to the membrane of the Golgi complex and differents organelles implicated in membranes trafic in the animal cell. Membranes fractions of the differents organelles were used to raise antisera which were then screened by indirrect immunofluorescence on tissue cultured cells and frozen,thin sections of tissues.There were many unwanted antibodies to others cell components and these were removed by suitables adsorptions steps wich were carried out until the pattern of fluorescence labeling was that expected for the organelle of interrest.Others antibodies were prepared against purified proteins or by using the monoclonal antibody technic.Electron microscopic studies were performed using immunoperoxidase labelling of tissue cultured cells or immunogold labelling of thin,frozen sections of tisues.the antigen reconized by the purified antibodies were characterized by immunoprecipitation or by immunoelectrotransfert (western blot).

Antibodies to the Golgi complex labelled the stacks of flattened cisternae which comprised the central feature of the complex. The polyclonal antibodies also labelled some lysosome structures, presumably during degradation.The antigen recognised was a polypeptide, (Mr 135 Kd), implicated in the transport of membrane proteins through the Golgi complex.

Anti-RER antibodies labelled only the endoplasmic reticulum including the nuclear membrane.They were largely dirrected against four polypeptides (Mr 29, 58, 66 and 91 Kd).

Anti-lysosome antibodies labelled the membrane of the lysosome The antigen was occasionaly detected in the Golgi complex.In some systems it was also detected at the plasma membrane and in endoctoitic structures such as coated vesicles.the antibodies recognised a single polypeptide (Mr 100 Kd) presumably the H+,K+,ATPase.

Anti-smooth endoplasmic reticulum were raised against epoxide hydrolase (Mr 56 Kd). The antibodies recognized the membranes of the smooth endoplasmic reticulum in rat liver.

Antibodies were raised against the high molecular weight component of clathrin (Mr 180 Kd).Polyclonal antibodies labelled coated pits and coated vesicles at the plasma membrane and in the Golgi complex.A monoclonal antibody did not labelled these structure but probably recognised a soluble pool of clathrin.

These differents antibodies have been used under a variety of physiological and experimental conditions where the morphology of the original structures was not recognizable.For example they proved useful for biochemical studies on subcellular fractions, particulary those from organelles such as the Golgi complex , which tend to lose their characteristic morphology during isolation. They were used to follow membrane reorganisation under specific stimulation conditions such as mitosis, secretion stimulation, hormones treatment etc.

More tools are now necessary to further dissect intracellular organelles. The production of new antibodies specific for each functional unit or displaying different fate upon stimulation conditions should prove extremely usefull in the understanding of the function of the intracellular organelles.

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212 THE ORGANIZATION OF GOLGI-ASSOCIATED GLYCOSYLATION MECHANISMS

Eric G. Berger, Dept of Pharmacology, Biocenter, University of Basel, CH-4056 Basel, Switzerland

Glycan biosynthesis constitutes one of the major biosynthetic mechanisms. In eucaryotic cells, it is restricted to the endomembranes of the secretory pathway (SP). The life cycle of a glycoprotein which moves along the SP is characterized by sequential glycosylation and processing steps until it arrives at its functional site which may be a subcompartment of the SP, the cell surface or the extracellular space. Our current research deals with the elucidation of the correct sequence of glycosylation steps along the SP. One of the enzymes involved in chain elongation of glycoprotein glycans, galactosyltransferase (GT), has been localized to the trans cisternae of the Golgi apparatus in HeLa cells and myotubes by immunocytochemistry and thus may constitute a useful model for topogenetic studies. The life cycle of this enzyme has been studied in HeLa cells using a pulse/chase protocol. These experiments inferred that GT moves within 20 to 60 min to the Golgi apparatus where it remains for a half-time of 19 h. Eventually, GT is exported through a constitutive secretory mechanism which implies a proteolytic cleavage of 2 kD, presumably the membrane spanning domain. In other cell types such as enterocytes, GT is expressed on the cell surface: Corresponding evidence was based on immunocytochemistry on tissue biopsy specimens and on CaCo-2 cells, a cell line derived from a human colon carcinoma. The localization of GT was compared with sialyltransferase (ST) by immunofluorescence in fibroblasts: ST appeared to be concentrated in vesicles clustered around the Golgi apparatus. This result provided support for the concept of a sequential compartmentalized glycosylation process. Thus, glycosyltransferases which are intrinsic components of the endomembrane system of the SP are restricted to defined subcompartments. Definition of the signals which retain the transferases at their site of action awaits further studies.

213 INTRACELLULAR TRANSPORT OF TWO ENDOMEMBRANE PROTEINS: GLUCOSIDASE II (ER) AND GALACTOSYLTRANSFERASE (GOLGI) . Ger J. Strous, Peter van Kerkhof, and Daniela Brada*, Laboratory of Cell Biology, University of Utrecht, Medical School, Nic.Beetsstraat 22, 3511 HG Utrecht, The Netherlands, and *Department of Biochemistry, University of California, Berkely, CA, USA

The mechanism of (glyco)protein transport between the rough endoplasmic reticulum and the Golgi remains largely unknown. Following the en bloc transfer of Glc3Man9GlcNAc2 precursor oligosaccharide from its dolichol pyrophosphoryl derivative to nascent polypeptide chains in the biosynthesis of N-linked glycoproteins, the oligosaccharides are modified by a sequence of reactions, generating N-glycans with either high mannose and/or complex type structures. Processing of the high mannose precursor is initiated by removal of the 3 glucose residues. They are removed in the endoplasmic reticulum by two enzymes, glucosidase I and II. The terminal alpha(1-2) linked glucose is removed by the enzyme glucosidase I, while the two inner alpha(1-3) residues are hydrolyzed by glucosidase II. Glucosidase II has been partially purified from rat liver and kidney (1-2). Brada and Dubach reported an apparent Mr on SDS-PAGE of about 100,000 for the subunits of the enzyme from rat liver. They also raised polyclonal antibodies against the kidney enzyme and were able to localize the enzyme in pig liver (3). Glucosidase II was present in the rough and the smouth ER, and in autophagosomes.

We have studied the biosynthesis and turnover of glucosidase II in a cell line derived from rat hepatocytes. Immunoprecipitation of the enzyme after metabolic labeling in the presence of (35S)methionine and analysis on SDS-PAGE revealed that the enzyme is synthesized as a Mr = 94,000 in these cells. Pulse labeling followed by prolonged chase periods did not change the apparent molecular weight. Digestion of the Mr = 93,000 band with endoglucosidase H lowered the Mr by about 1500, indicative for the presence of one N-linked oligosaccharide. Upon longer chase times (2-3 h) glucosidase II did not become resistant to digestion by the glycosidase, indicating that N-linked oligosaccharide chain of the enzyme remains in the high mannose configuration. Molecular weight analysis by gel filtration on Biogel P-4 of the N-linked oligosaccharide showed that the glycan is most probably present as Glc1Man9GlcNAc2. This suggests that glucosidase II cleaves only the penultimate glucose in its own oligosaccharide chain. It also shows that the enzyme does not reach the Golgi complex. The half-life of the enzyme is in the order of 60 min, which is extremely short for this kind of protein.

As the turnover of the enzyme seems rather short as compared to comparable membrane enzymes of the rough endoplasmic reticulum, we have examined the effect of inhibitors of lysosomal degradation. Leupeptin had only little effect on the turnover of glucosidase II. However, low concentrations of primaquine, (causing elevation of the pH in acidic compartments) prolonged the life-time of the enzyme by a factor of two. Furthermore, we studied the influence of the N-linked oligosaccharide chain on maturation and life-time of the enzyme, using tunicamycin, and (methyl)deoxynoijirimycin. Growing the cells in the presence of tunicamycin extended the life-time of the enzyme considerably.

The processing and turnover of the Golgi enzyme galactosyltransferase differs in many aspects from that of glucosidase II. Galactosyltransferase belongs to the glycosyltransferases. In several tissues and cell lines the enzyme is localized by immunocytochemistry to the two or three trans-cisternae of the Golgi complex and may thus be considered a specific membrane component of this type of endomembrane (4, 5). As a consequence it is the most common Golgi "marker" enzyme in cell fractionation studies. Study of its biosynthesis, membrane orientation and turn-over in several tissues and cultured cell lines has broadened our knowledge about Golgi function itself. The enzyme is oriented towards the lumen of the cisternal space. In this orientation it catalyzes the transfer of galactose to glycoprotein-bound acetylglucosamine and, in the presence of alpha-lactalbumin, to glucose as shown in the Golgi complex of mammary gland epithelial cells (6). The enzymatic properties of galactosyltransferase are well known. The metabolism of galactosyltransferase has been extensively studied in HeLa and HepG2 cells (7, 8). The enzyme is synthesized in the rough endoplasmic reticulum and provided with one N-linked oligosaccharide and palmitate residues. In the Golgi complex terminal sugars are attached to the N-linked oligosaccharide and extensive O-glycosylation takes place. Comparison of the biosynthesis of galactosyltransferase in human cell lines derived from hepatocytes and breast cancer cells revealed differences in glycosylation. The half-life of the enzyme is about 20 h, whereafter a soluble form appears in the culture medium. Release of galactosyltransferase into the medium is observed in all cell lines studied.

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214 ALPHAVIRUS AND BUNYAVIRUS GLYCOPROTEINS AS MARKERS OF THE EXOCYTIC TRANSPORT PATHWAY.
Jaakko Saraste(1), Esa Kuismanen(2), Nina Gahmberg(2), and Ralf Pettersson(2). (1) Department of Virology and (2) Recombinant DNA Laboratory, University of Helsinki, Helsinki, Finland.

We have used immunolocalization in light and electron microscopy in combination with biochemical techniques to study the intracellular transport of the membrane glycoproteins of the alphavirus Semliki Forest Virus (SFV) and the bunyavirus Uukuniemi virus (UUKU) in BHK-21 cells. These two enveloped viruses differ in their cellular site of maturation: Whereas the budding of SFV occurs at the plasma membrane, where the virus glycoproteins are transported and concentrated, UUKU matures intracellularly at the membranes of the Golgi complex. The UUKU particles are released from the cells by fusion of virus-containing transport vesicles with the plasma membrane. We have compared the morphological transport pathway of the membrane glycoproteins specified by these two viruses in order to obtain information of the cellular transport processes which operate in the routing of the virus glycoproteins and which, at least partly, determine the cellular site of virus maturation.

To study in detail the transport of SFV p62/E1 glycoprotein complex, a temperature-sensitive mutant (ts-1) which allows the synchronization of protein transport between the rough ER and the cell surface, was used. The proteins were localized in saponin-permeabilized cells using a pre-embedding immunoperoxidase procedure (1). In addition, incubation at reduced temperatures (15°C and 20°C) was carried out to arrest protein transport at distinct sites along the transport pathway. The findings of these immunoelectron microscopic studies (2) can be summarized as follows: SFV membrane glycoproteins are transported from the rough ER in small (about 100 nm) vesicles which carry the proteins to the Golgi region. These primary transport vesicles apparently fuse to form larger vesicles which function in the entry of the proteins into the Golgi complex. At 15°C the proteins are slowly transported from the rough ER but they accumulate in the pre-Golgi vesicles and do not enter the cisternal elements of the Golgi complex. Reduced temperature apparently affects a fusion step required for the entry into the cis-Golgi cisternae. During uninhibited transport the proteins move across the Golgi complex in cis to trans direction (1). Transport across the Golgi also occurs at 20°C but the SFV membrane proteins fail to appear at the cell surface at this temperature although they are terminally glycosylated as shown by aquisition of resistance to Endo H. Immunoelectron microscopy demonstrated that the proteins accumulate at 20°C in trans-Golgi cisternae as well as in vacuoles and vesicles in the trans-Golgi region. The virus glycoproteins were also localized in about 200 nm vesicles present in peripheral cytoplasm. It is likely that these vesicles represent the carriers which transport the proteins from the juxtanuclear Golgi region to the cell surface.

Since at 15°C the proteins could be arrested at the level of an intermediate compartment between ER and the Golgi complex, temperature shift experiments were carried out to investigate the pathway of the proteins across the Golgi apparatus. Shortly after reversal of the temperature from 15°C to 37°C (or 20°C) the virus proteins could be localized in all of the Golgi cisternae indicating that transport across the Golgi occurs rapidly. In these syncronized conditions no labeling of the 50-80 nm vesicles of the Golgi periphery could be detected suggesting that the cisternal elements of the Golgi apparatus as entities carry the virus membrane proteins through the Golgi stack.

In contrast to SFV glycoproteins, the two membrane proteins, G1 and G2, of UUKU appear to be transported separately from the rough ER to the Golgi apparatus and form a complex just before incorporation into virus particles. Immunolocalization methods have demonstrated the gradual accumulation of the UUKU glycoproteins in the Golgi complex during infection (3). At the midcycle of infection progressive vacuolization of the Golgi complex takes place. This apparently is one of the prerequisites for the entry of the virus nucleocapsids into the Golgi region. Recent studies using a temperature-sensitive mutant (ts-12) of UUKU have shown that the vacuolization occurs in the absence of virus budding suggesting that the vacuolization is the function of the virus glycoproteins which accumulate in the Golgi complex (4). UUKU virions bud into the lumen of distended Golgi cisternae, are packaged into large transport vesicles which carry the virus particles to the cell surface.Some virus glycoproteins remain associated with the limiting membrane of these transport vesicles and reach the plasma membrane as the vesicles fuse with the cell surface and release their content virus particles. The expression of UUKU glycoproteins at the plasma membrane, in contrast to e.g. SFV glycoproteins, is insensitive to monensin (5). Monensin, however, efficiently inhibits the assembly of the virus in the Golgi complex.

In addition to virus particles and distended cisternal membranes of the Golgi complex, UUKU membrane proteins could be localized in 50-80 nm peripheral Golgi vesicles, which are present in large numbers in the Golgi region of the infected cells. If the UUKU-infected cells are treated with cycloheximide during the midcycle of infection (between 6 and 10 hr), virus budding is greatly diminished as shown by electron microscopy.Transport of the virus glycoproteins to the cell surface is inhibited and the proteins remain in the Golgi complex. In the Golgi apparatus the UUKU glycoproteins were localized by immunoelectron microscopy in the cisternal membranes as well as in the 50-80 nm Golgi vesicles which are also labeled in control cells. These findings suggest that the small peripheral vesicles may function in the recycling of the virus membrane proteins within the Golgi complex to facilitate the accumulation of the proteins, the vacuolization of the Golgi membranes, and the maturation of the virus at this site.

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215 ACTIVE AND LATENT ENZYMES IN THE ADENOHYPOPHYSEAL CELLS OF THE RAT.
A.J.Nevorotin. 1st Medical Institute, Leningrad, U.S.S.R.

Pituitary glands of female rats were either (1) immediately fixed, or (2) incubated before the fixation in the highly osmomolar medium (A.J.Nevorotin. 1980. J.Cell Sci., 46, 433-454) for 1 hr at 37°C, or (3) postfixationally treated with Triton X-100 or saponin, or with (4) potassium borohydride solutions. The specimens were further processed for acid phosphatase (AcPase), aryl sulfatase (ArSase), or thiamine pyrophosphatase (TPPase). After (1) both AcPase and ArSase were localized to the lysosomal domain of mammotrophs and somatotrophs while TPPase was mainly restricted to the trans-pole of the Golgi apparatus (GA). In the case (2) the three enzymes, in addition to these sites, could be easily found throughout the GA and the cisternae of the rough endoplasmic reticulum (RER). Since both the membrane labilizers (3) and the potent reducing agent (4) known to restore glutaraldehyde-damaged tertiary protein structure (W.D.Eldred et al. 1983. J. Histochem. Cytochem., 31, 285-292) also resulted in somewhat analogous changes in the enzyme reactivity of GA and RER, it is suggested that AcPase, ArSase and TPPase found in the series (2-4) outside their usual sites may represent their latent (catalytically inactive) fractions. To what extent that latency is due to conformational alterations in the enzyme molecules following the fixation and/or histochemical procedures employed, or it might reflect some intrinsic catalytic immaturity of the hydrolases at the early stages of their biogenesis, is not clear.

216 A CELL-FREE ASSAY FOR AN EXOCYTIC FUSION EVENT
Philip G. Woodman and J. Michael Edwardson
Department of Pharmacology, University of Cambridge, Hills Road, Cambridge CB2 2QD, U K.

In eukaryotic cells newly synthesised plasma membrane proteins are delivered to their destination by vesicle fusion. In order to investigate the nature of this event we have developed a cell-free assay for fusion which exploits the enzyme activity of influenza virus neuraminidase (NA).

Semliki Forest virus (SFV) with envelope proteins containing [^{3}H] sialic acid was bound to the plasma membranes of baby hamster kidney (BHK) cells. The cells were then homogenised and a post-nuclear supernatant prepared. Another extract was prepared similarly from BHK cells infected with influenza virus and containing NA en route to the plasma membrane. Fusion between vesicles from these two extracts, termed acceptor and donor extracts respectively, results in access of NA to [^{3}H] SFV and release of free [^{3}H] sialic acid. This release was detected through transfer of radioactivity from a trichloroacetic acid (TCA)-insoluble to a TCA-soluble fraction.

Both ATP-dependent and ATP-independent components of release of free [^{3}H] sialic acid were detected. The ATP-dependent component appears to be a result of fusion between sealed vesicles, since it is resistant to the addition to the incubation mix of 2,3-dehydro-2-desoxy-sialic acid, an inhibitor of NA which cannot penetrate membranes. This component is maximal when donor extracts are prepared from cells in which NA has been concentrated between the Golgi complex and the plasma membrane, which indicates that the fusion event is specific. At present we are attempting to characterise this component further.

217 THE DISTRIBUTION IN THE GOLGI COMPLEX OF PSA- AND LCA-BINDING SITES
Margit Pavelka and Adolf Ellinger, Institute of Micromorphology and Electronmicroscopy, Schwarzspanierstrasse 17, 1090 Vienna, Austria

By means of a pre-embedment technique and using peroxidase conjugates of the lectins (1), we studied the location in Golgi subcompartments of binding sites for the *Pisum sativum* (PSA) and *Lens culinaris* (LCA) *lectins*; both *mannose/glucose* specific lectins require a *fucose* residue attached to the *asparagine-linked N-acetyl-glucosamine of glycopeptides* (2,3), and hence, preferentially bind with *core-fucosylated N-linked oligosaccharides*. In all cell types studied, covering small intestinal absorptive and goblet cells, pancreatic acinar cells and fibroblasts, both lectins caused intense reaction of cis and/or medial cisternae of the Golgi stacks, the penultimate cis cisterna being the most constantly labeled one. In the acinar cells, the reactions were confined to the cis/medial cisternae; in the other cell types, PSA and LCA label, in addition, was found in limited segments of cisternae of the trans Golgi side, especially of the transmost one. *Fucosidase pre-treatment* experiments revealed clearly diminished PSA/LCA staining; being in line with biochemical results (2,3), this confirms that fucose is an important determinant also for the cytochemical PSA/LCA reactions shown here.

The present results indicate that a class of *glycopeptides with core-fucosylated N-linked oligosaccharides* is preferentially concentrated in *cis and medial Golgi cisternae, the penultimate cis cisterna being favoured.*

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218 STRUCTURE AND REGULATION OF CONTACT SITES BETWEEN OUTER AND INNER MEMBRANE OF MITOCHONDRIA.
A.J. Verkleij and G. Knoll. Institute of Molecular Biology, State University of Utrecht, Padualaan 8, NL-3584 CH Utrecht, The Netherlands.

Distinct contact sites between inner and outer membranes of isolated mitochondria have been suggested from thin section electron micrographs (1), spots of diminished negative surface charge allowing the close apposition (2). This surface charge over the bulk areas of the two membranes is thought (2) to be the reason for the usually observed intermembrane space when standard preparation procedures are applied (3).

However classical preparation procedures do not allow such conclusions since chemical fixation and dehydration induce severe artefacts. By reducing the denaturating effects of the conventional methods in a series of investigations it was shown that both membranes of mitochondria in situ are closely apposed (e.g. 4). This is in agreement with early results obtained by rapid freezing - seen today as the only reliable fixation method for biological specimens - and following freeze substitution avoiding any chemical pretreatment (5).

Freeze fractured mitochondria - rapid frozen in vivo - reveal frequent jumps or deflections of the fracture plane between the inner and outer membranes (6). This high frequency of fracture plane deflections was also found with isolated well coupled mitochondria during oxidative phosphorylation (7,8). Only few deflections are observed with mitochondria frozen in other experimental states, namely without ADP but with substrate or without both substrate and ADP (7,8). The phenomenon is totally abolished by chemical pretreatments (as normally used for freeze fracture preparations) or uncoupling of the mitochondria (7,8). Freeze substitution shows that the membranes of mitochondria exhibiting high frequencies of fracture plane jumps are closely apposed whereas a low frequency is correlated with a clear intermembrane space (6).

This characteristic morphology of coupled and in vivo mitochondria, revealing frequent fracture jumps, can also be induced in isolated mitochondria after incubation with Ca^{++}, Mn^{++}, Mg^{++}, apocytochrome C and at 37°C (9). From these results it has been hypothesized that the fracture jumps represent contact points between inner and outer membranes, where the two membranes are joined (semifusion) by locally and temporarily exposure of non bilayer behaviour of membrane lipids (9). This because it has been demonstrated that bivalent cations, increased temperature and apocytochrome C are factors which induce nonbilayer conformation of lipids isolated from mitochondria. Adriamycin which competes with divalent cations for binding with negatively charged phospholipids and depresses the nonbilayer behaviour of lipid in mitochondria reduces the contact sites as seen with freeze fracturing (10).

Biochemical evidence supporting the proposal of contacts between the inner and outer membrane has been put forward from different points of view. So it is assumed that ATP-ADP translocations occur via such contact sites (11). Moreover the translocation of inner mitochondrial protein such as apocytochrome C, which are synthesized on free ribosomes has been explained by the presence of contact sites (12). At last, facilitated transport of lipid molecules from inner to outer membrane as found in the presence of Ca^{++} (13) are in line with local semifusion of both membranes.

In conclusion, morphological and biochemical evidence is growing, which support the view that there are apolar contacts in dependency of physiological conditions between the outer and inner membrane of the mitochondria.

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219 FUNCTIONS IN METABOLIC REGULATION OF CONTACT SITES BETWEEN MITOCHONDRIAL BOUNDARY MEMBRANES.

D.Brdiczka, I.Riesinger and V.Adams. Faculty of Biology, University of Konstanz
PO Box 5560, D-7750 Konstanz 1, F.R.G.

The number of contacts between mitochondrial boundary membranes as visualized by freeze fracture, correlates with the degree of coupling between electron transport and phosphorylation. Thus, contact frequency is high in phosphorylating mitochondria, but very low after uncoupling (1). The absolute number of contacts in phosphorylating mitochondria appears to be under hormonal control. In cultured hepatocytes, contacts significantly increase with epinephrine (acting as an alpha-1-agonist with Ca2+ as a second messenger), whereas glucagon (ß-agonist, C-AMP) causes a decrease in the frequency of boundary membrane contacts (2). Since glucagon does not uncouple mitochondria, it appears the contact sites are important in the regulation of metabolite exchange but not in the regulation of coupling. The formation of contacts during phosphorylation may be a signal for mitochondrial competence in ATP/ADP and substrate exchange, with Ca2+ regulating the contact frequency. This view is supported by the observation of a functional coupling of hexokinase to the inner mitochondrial compartment (3). In this case, intramitochondrial ADP is supplied by a peripheral bound kinase which is functionally coupled to the adenine translocator. The high energy phosphate is transferred to the cytosol as glucose-6-phosphate.

We have studied the functional coupling of hexokinase in more detail. Hexokinase specifically binds to the pore in the outer membrane (4). Isolation of the contact sites and electronmicroscopy (5) have revealed that this enzyme preferentially binds to the pore in the contact region. Cross-linking studies suggest an oligomeric structure (dimer or trimer) of the pore in the contact area. Applying a new cell extraction technique to isolated hepatocytes, we observed that 80% of Isoenzymes I-III are bound to the mitochondrial fraction. The binding of these three isoenzymes appears to be regulated by metabolites (such as FFA) and/or hormones because the amount of bound hexokinase decreases following: 1) incubation of cells with FFA (1) and, 2) C-AMP dependent phosphorylation of a 14 kDa polypeptide at the mitochondrial surface. Therefore, hexokinase binding may be regulated by: 1) changes in the frequency of contact sites between the boundary membranes and 2) changes in the negative surface charge.

Hexokinase can be considered as an "ambiquitous" enzyme which changes its localization according to the metabolic state of the cell. The enzyme may either be linked to glycolysis or oxidative phosphorylation. Both of these ATP-providing systems are regulated by changes in the phosphorylation potential. A high phosphorylation potential in the cytosol reduces the rate of glycolysis in favour of the more efficient endoxidation of pyruvate in the mitochondria. Indeed, the phosphorylation potential is significantly higher in the cytosol in the presence of glucose. To explain this difference, it is assumed that the adenine translocator becomes displaced from equilibrium by the transmembrane potential across the inner membrane (6). However, because the translocator has the same binding constants for ATP and ADP, a high ATP/ADP ratio in the cytosol may considerably lower the effective rate of the nucleotide transport system. Therefore, it may be not the extramitochondrial free ADP, but the intramitochondrial ATP/ADP ratio which regulates oxidative phosphorylation (7). Concerning the intramitochondrial ADP supply, a functional coupling of peripheral kinases to the inner compartment is important. This has been demonstrated recently in highly glycolytic tumor cells, where no coupling of the bound hexokinase to the inner compartment was observed. (8). Conversely, bound hexokinase in liver mitochondria preferentially uses ATP provided by oxidative phosphorylation (9). Electronmicroscopic analysis of mitochondria in tumor cells has revealed a significant decrease in contact sites and suggests that contacts play a role in the functional coupling of hexokinase (2).

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220 **CONTACT SITES AND PROTEIN TRANSLOCATION IN MITOCHONDRIA**

M. Schleyer, M. Schwaiger, F.-U. Hartl and **W. Neupert.** Institut für Physiologische Chemie, University of Munich, Goethestr. 33, D-8000 München 2, FRG

The vast majority of mitochondrial proteins are synthesized in the cytosol and posttranslationally translocated into mitochondria. In many cases these proteins have to be transported across two membranes; the outer and inner mitochondrial membranes. The question will be addressed as to whether this transport across both membranes occurs in a single step or whether these two membranes are crossed in two consecutive reactions. Data will be provided which shows that translocational intermediates of precursor proteins (ATPase subunit 2; cytochrome c_1) can be trapped. The amino termini of these translocational intermediates have been transfered into the matrix space, whereas the major portion of the molecules are still on the surface of the outer membrane. Trapping of the intermediates could be achieved i) by lowering the temperature during import to about 8°C; or ii) by prebinding the precursors to antibodies raised against the mature proteins. In case i), the intermediates can be chased into the matrix by raising the temperature to 25°C. A membrane potential was found to be required only for the translocation to the intermediate state, but not for the subsequent step of complete internalization. These results led us to postulate the existence of "translocation contact sites". We will report on attempts to correlate these biochemical findings with structural studies on the interaction of outer and inner mitochondrial membranes.

N. Pfanner and W. Neupert, EMBO J. **4** (1985) 2819-2825.
M. Schleyer and W. Neupert, Cell **43** (1985) 339-350.

221 DENSE MATRIX GRANULES AND CONTACT SITES IN MITOCHONDRIA.

W.A. Jacob. Department of Medicine, University of Antwerp, UIA, B-2610 Wilrijk, Belgium.

Subarachnoid haemorhage and intravenous injection of noradrenalin cause the migration of mitochondrial matrix granules of the myocardium, towards the periphery of the organel. Here they loose gradually their intrinsic electron density and vanish completely when the heart is strongly stimulated (1). Blocking of electron transport "stabilizes" the very labile native granules. Treatment with KCN allows the isolation of mitochondria from heart tissue, still containing native granules (2). Amytal promotes the occurance of much larger granules. Consecutive treatment with amytal and noradrenalin causes no migration of the granules, but still makes them loose electron density, leaving in the matrix space membrane bound vesicles. The lipid and calcium content of the granules is demonstrated by cytochemical methods (3). Concomitant with the disappearance of the granules, an important increase of matrix calcium is measured with a laser microprobe technique. This high calcium level creates conditions for fusion between inner and outer membrane to occur. These fusion zones are demonstrated ultrastructurally by carefully adapted fixation procedures. It is also shown that the matrix granules are closely linked to these processes, possibly being incorporated partially in the inner membrane. The substantial increase of intramembrane particle content of the inner membrane upon energization may add to this possibility (4). It is hypothesized that matrix granules constitute a buffer compartment by which transport processes are modulated in energized heart mitochondria.

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222 **CONTACT SITES AND IMPORT OF MITOCHONDRIAL PROTEINS: A MORPHOLOGICAL STUDY. M. Schwaiger,** V. Herzog and W. Neupert. Institut für Physiologische Chemie u. Zellbiologie der Universität München, Goethestr. 33, 8 München 2, FRG

In a number of instances protein topogenesis involves translocation across two membranes. The general question is raised as to whether this process follows a two-step mechanism or whether the polypeptide traverses the two membranes in a single step. Recent experiments have suggested that a number of proteins are imported by a one-step mechanism. By binding precursor proteins to specific antibodies before import, stable membrane spanning intermediates could be generated. (M. Schleyer and W. Neupert, Cell **43** (1985) 339-350).

We have attempted to localize the translocation sites on isolated mitochondria of **Neurospora crassa.** Mitochondria containing translocational intermediates of ATPase SU2 stabilized by the prebound antibody were labelled with 5 nm gold particles conjugated to protein A. The protein-A gold label was restricted to areas of the outer membrane in close contact with the inner membrane. If the outer membrane is partially removed from mitochondria by shearing, breakage occurs to a significant extent at, or close to, the contact sites, suggesting that they are stable structures. Upon incubation with reticulocyte lysate some mitochondria lose part of their outer membrane and the inner membrane becomes accessible to precursor proteins. Again the gold label can only be found at the contact site, but not at the inner membrane.

The observations are interpreted to mean: i) Import of proteins which require a membrane potential occurs specifically at the contact sites and ii) Import across the two membranes is not a two-step event with a soluble intermediate in the intermembrane space.

223 STUDIES OF THE RELATIONSHIP BETWEEN THE INNER AND OUTER MEMBRANES OF RAT LIVER MITOCHONDRIA AS DETERMINED BY SUBFRACTIONATION WITH DIGITONIN

Vera JANCSIK /1/, Monica LINDEN /2/, Latifa DORBANI /3/, Alvaro RENDON /3/ and B.Dean NELSON /2/

/1/ Institute of Enzymology BRC,Hung.Acad.Sci.,Budapest,Hungary; /2/ Arrhenius Laboratory Stockholm University, Sweden; /3/ Centre de Neurochimie du CNRS, Strasbourg,France

Points of contact or fusion between the outer and inner mitochondrial membranes have been reported by several investigators. In order to learn more about the composition of such membrane domains, we have attempted to subfractionate the outer membrane/inter membrane space compartment of rat liver mitochondria with digitonin. Porin was followed immunologically as a unique marker of the outer membrane. Porin was removed concomitantly with monoamine oxidase, suggesting that the outer membrane is removed as a single unit with no apparent association to the inner membrane. The membrane environment of porin in liver mitochondria differs from that found in rat brain. Incubation and fractionation of liver mitochondria under different conditions of energization did not alter the relationship between the two membranes by these criteria. In contrast, Ca^{++} /100 nmoles/mg protein/ promoted the release of malate dehydrogenase together with the outer membrane markers. Our results are in favor of a possible role of cholesterol domains in the interaction between the inner and outer membranes.

224 SUBFRACTIONATION OF THE OUTER MEMBRANE OF RAT BRAIN MITOCHONDRIA. EVIDENCE FOR THE EXISTENCE OF A DOMAIN CONTAINING THE PORIN/HEXOKINASE COMPLEX

Latifa DORBANI /1/, Vera JANCSIK /2/, Monica LINDEN /3/, B.Dean NELSON /3/, and Alvaro RENDON /1/

/1/ Centre de Neurochimie du CNRS, Strasbourg, France; /2/ Institute of Enzymology,BRC Hungarian Academy of Sciences,Budapest,Hungary; /3/ Arrhenius Laboratory, Stockholm University, Sweden

Isolated and well characterized rat brain non-synaptic mitochondria were subfractionated by digitonin. Antibodies to a uniquely outer membrane protein, porin, has allowed us to use this protein for the first time as an outer membrane marker in brain. Hexokinase, which binds to porin, was also measured. Based upon the sequential release of these and other marker enzymes with increasing concentrations of digitonin, three outer membrane domains have been identified. Two populations of porin were found which seem to exist in different membrane environments with regard to cholesterol. One of these populations binds most of the hexokinase and is located in an environment which is relatively free of cholesterol and which appears to be intimately associated with the inner membrane. It is proposed that the porin/hexokinase complex in brain mitochondria is located in a cholesterol free membrane domain together with inner membrane components. This domain has the features of contact points which have been visualized by electron microscopy.

225 EFFECT OF CHLOROPHILL ON THE FORMATION OF MEMBRANE JUNCTIONS IN LIPOSOMES COMPOSED OF GALACTOLIPIDS AND PHOSPHATIDYLGLYCEROL. I.A.Vasilenko (1), Yu.S.Tarakhovsky (2), V.L.Borovyagin (2). (1) Lomonosov Institute of Fine Chemical Technology, Moscow, (2) Institute of Biological Physics, USSR Academy of Sciences, Pushchino, USSR.

Chlorophill was shown to induce structural changes in bilayer liposomes composed of galactolipids/phosphatidylglycerol mixtures and in liposomes composed oftotal lipids from spinach thylacoids at the molar ratio of lipid/pigment = 1/5 in both. As a result of these changes the appearence of intramembrane lipidic particles and the formation of hexagonal H_{II}-phase was observed in both types of liposomes. The formation of linear contacts, of inverted lipid micelles, the fusion of monolayers of contacting membranes, and the formation of stalk and pore structures are described as initial stage of structural changes. The inverted micelles are formed as a result of fusion of adjacent bilayers in the sites of their point and/or circular contacts. The formation of these contacts requires at least two bilayers. The formation of multiple contacts of similar nature was confirmed for tightly packed bilayers. As evidenced by the results obtained, structural properties of individual lipid species may be significantly altered when they are in mixtures with other lipids.

226 CELL SURFACE DYNAMICS: MOBILITY AND FUNCTION OF MEMBRANE PROTEINS AND LIPIDS.
L. Mátyus(1), Margit Balázs(1), J. Szöllősi (1), J. Matkó(1), M.J. Fulwyler(2), L. Trón(1), A. Aszalós(3), S. Damjanovich(1). (1) Department of Biophysics, University Medical School of Debrecen, H-4012 Debrecen, POB 3, (2) Lab. Medicine, N-531 UCSF, San Francisco CA 94143, and (3) Food and Drug Administration, HFN 172, Washington, DC 20204

Dynamic properties of membrane lipids and proteins of human and mouse lymphocytes have been studied with a variety of biophysical technics: fluorescence recovery after photobleaching, flow cytometric energy transfer, membrane potential measurements with carbocyanine dyes and titrating the accessibility of SH groups in combination with drugs and ionophores. Proximity and lateral mobility of different surface elements (like MHC proteins, ligand binding sites characterising lymphocyte subpopulations, and also membrane lipids) were determined. The changes in membrane potential upon addition of ionophores, antibodies, and - among others - cyclosporin A were studied in a flow cytometer by the aid of carbocyanine dyes. The observed changes were considered like those monitoring dynamic behavior of cell surface elements. Titration of cell surface SH groups with several SH reagents (changing their spectroscopic properties upon binding the available free or moderately masked SH groups) revealed changes in accessibility of free or masked SH groups as shown by the kinetics of their reaction with other specific SH reagents or depending upon the hyper- or hypopolarized state of the cytoplasmic membrane.

227 SIMULTANEOUS DETECTION OF LYMPHOCYTE DIFFERENTIATION ANTIGENS IN AIDS-VIRUS INFECTED CELLS BY FLOW CYTOMETRY.
Daniel P. Stites(1), Thomas McHugh(1), Conrad Casavant(1), John Krowka(1), Richard Rodgers(1), Andrew Moss(2). (1) Dept. of Laboratory Medicine, and (2) Dept. of Epidemiology and International Health, University of California, San Francisco, CA, USA.

We investigated expression of various leukocyte membrane differentiation antigens in AIDS virus infected and control subjects with monoclonal antibodies (Mabs) and flow cytometry. Simultaneous two color immunofluorescence with phycoerythrin (PE) or fluorescein isothiocyanate (FITC) coupled Mabs was done in a FACS analyzer. Monocytes were gated out by double labelling with Mab-Leu M3 PE + FITC. A rank order of association of leukocyte phenotype changes was observed which correlated with virus seropositivity; H/S>T suppressor/cytotoxic>T cytotoxic>Leu 2+7+>T helper cells. Simultaneous 2 color immunofluorescence allowed for identification of helper inducer (CD4) and suppressor/cytotoxic (CD8) cells. Results show that increased killer but not suppressor T cells, and reduction in suppressor/inducer, not helper T cells accounts for inverted helper/suppressor ratios characteristic of the infection. Striking increase in expression of MHC-Class II (DR and DQ) antigens was observed in virus infected cell lines, Hut 78 and H9. These MHC antigens appear closely linked to viral proteins detected by anti-viral antibodies. Comparison of genomic response of HTLV-III, ARV, and LAV env-gp shows no homology with MHC Class II antigens. These results suggest immunodeficiency in AIDS virus infection is due to profound disturbance in many leukocyte subsets and is further related to possible autoimmunity to HLA antigens.

228 DISTRIBUTION OF HLA I AND HLA II MOLECULES ON THE CELL SURFACE. A FLOW CYTOMETRIC STUDY.
J. Szöllősi(1), M.J. Fulwyler(2), S. Damjanovich(1). (1) Department of Biophysics, University Medical School of Debrecen, H-4012 Debrecen, POB 3., (2) Lab. Medicine, N-531 UCSF, San Francisco CA 94143

The Flow Cytometric Energy Transfer (FCET) method, (Biophys. J., 45, 939-946. 1984) was used for the determination, on a cell-by-cell basis, of Fluorescence Resonance Energy Transfer (FRET) between labeled specific sites of cell surface constituents using a flow cytometer capable of dual wavelength excitation. FRET efficiency was measured between fluorescein- and tetramethylrhodamine-conjugated monoclonal antibodies bound to HLA I and HLA II antigens on the cytoplasmic membrane of Epstein-Barr virus transformed human B cells designated as PGF cells. While the HLA I antigens are distributed mainly in monomeric form on the cell surface, the HLA II antigens form dimers or higher polymers as determined from the FRET efficiency data. Significant FRET efficiency was detected between the HLA I and HLA II molecules suggesting, that they are associated on the cell surface.

229 FLOW SORTING OF X AND Y CHROMOSOME BEARING SPERM INTO SEPARATE POPULATIONS ON THE BASIS OF DNA CONTENT. L.A. Johnson US Dept. of Agriculture, Agrigultural Research Service, Reproduction Laboratory, Beltsville, MD, USA

An orthogonal flow cytometer/cell sorter was modified to control the orientation of sperm as they intersect the laser beam. This enabled the high resolution measurement of sperm DNA content. An Epics V flow cytometer/cell sorter was modified by beveling the sample injection tube tip and by adding a second fluorescent detector located forward to the laser beam. Sperm were washed and fixed in 80 % ethanol. Prior to staining the fixed sperm were treated with papain and dithioerythritol. The sperm were then stained with Hoechst 33342 and analyzed for relative DNA content. Peaks representing the X and Y chromosome bearing sperm were resolved for cattle, sheep, swine, chinchilla, vole and several other species. Differences in DNA content between the X and Y chromosome-bearing sperm ranged from 3.4 % to 9.1 % for the various species. Coefficients of variation ranged from 1.1 to 1.4. Sperm from the creeping vole and the chinchilla have been sorted into separate populations and reanalyzed to verify the DNA content of the sorted X and Y sperm. A purity greater than 95 % was attained. The isolation of X and Y bearing sperm may aid in the discovery of a marker other than DNA which identifies either X or Y. Such information could aid the development of a practical procedure to preselect sperm for gender.

230 FLOW CYTOFLUOROMETRIC ANALYSIS OF ENDOTHELIAL CELL HETEROGENEITY IN HUMAN ATHEROSCLEROTIC AORTA.

V.S. Repin, A.S. Antonov, M.A. Nikolaeva and Y.A. Romanov
USSR Cardiology Research Center, Moscow, U.S.S.R.

In the primary culture endothelial cells (EC) harvested by dispase from human atherosclerotic aorta preserve morphological and functional heterogeneity they have in situ. The EC heterogeneity was evaluated in respect to DNA, protein content, DNA-protein distribution. Flow cytometry of the propidium iodide stained EC revealed that the share of 4c and more than 4c cells was significantly higher in lesioned area as compared to nonaffected vessels. For EC from human umbilical vein the distribution of FITC stained cells approached to normal (CV=47%, coefficient of assymetry, A_s=0.3). For EC from unaffected human aorta CV=55%, and A_s=0.5. For EC from human atherosclerotic aorta CV=73% and A_s=1.1. A shift in distribution accounted for an increased share of protein-rich 2c, 4c and hypertetraploid EC.

231 CELLULAR MEMBRANE DYNAMICS ARE EFFECTED BY MICROTUBULE DEPOLYMERIZATION.

A. Aszalós(1), M.M. Gottesman(2), and S. Damjanovich(3). (1) Division of Drug Biology, Food and Drug Administration, Washington DC 20204, (2) National Cancer Institute, NIH, Bethesda, MD 20205, and (3) Department of Biophysics, University Medical School of Debrecen, H-4012 Debrecen, POB 3.

Three lines of evidence were obtained indicating that microtubule depolymerization affects the function and the dynamics of the plasma membrane. The first evidence was obtained with a membrane potential sensing dye and with the use of flow cytometry. Microtubule depolymerization with Colcemid (0.5 µg/ml), colchicine (2.5 µg/ml) and vincristine (2.5 µg/ml) resulted in membrane depolarization in chinese hamster ovary cells. Evidence that the observed effect is due to microtubule depolymerization are as follows: 1.) effects were time dependent, 2.) required the entry of the drugs into the cell as indicated by the lack of membrane depolarization in a multi-drug resistent mutant, 3.) Colcemid-resistent tubuline mutant did not show membrane potential changes and 4.) taxol, the microtubule stabilyzing drug, prevented the action of the depolymerizing drugs. The second line of evidence comes from ESR studies using maleimid spine probes attached to proteins in the membrane. ESR probing the lipid domain of plasma membranes gave additional evidence. These studies indicate that the motional freedom of the probes, as expressed by the order parameter S is higher in microtubule depolymerized cell membranes than those of untreated cells. Our finding indicate that microtubule affecting drugs influence the function of cells via changes in plasma membrane functions.

232 INTERACTION OF THE B SUBUNIT OF CHOLERA TOXIN WITH ENDOGENOUS GANGLIOSIDE G_{M1} INFLUENCES LYMPHOCYTE MEMBRANE POTENTIAL. S.A. Mulhern (1), P.H. Fishman (2) and S. Spiegel (2). (1) Food and Drug Administration, Washington, D.C. and (2) National Insititute of Neurological and Communicative Disorders and Stroke, Bethesda, Maryland.

We have used the fluorescent cationic dye dihexyloxacarbocyanine and flow cytometry to monitor changes in the membrane potential of rat thymocytes exposed to the B subunit of cholera toxin. Recently, B was found to be mitogenic for rat thymocytes (Spiegel et al., Science 230: 1258, 1985) and changes in membrane potential may be an early event in proliferation. B caused a decrease in the membrane potential of the thymocytes in a time, temperature and dose dependent manner. Depolarization occurred after a lag of 20-30 min at 37°C with 20 nM of B. No increase in mitochondrial membrane potential was observed. Further studies carried out in the presence of oubain suggest that B is inducing an increase in the permeability of the plasma membrane to small ions. As the B subunit of cholera toxin is multivalent and binds exclusively to ganglioside G_{M1}, crosslinking of several gangliosides on the cell surface by B may modulate ion channels in the plasma membrane. A potential role for endogenous gangliosides in regulating membrane permeability has important implications for the function of gangliosides in various cellular phenomena such as growth, differentiation and electrical excitability of neurons.

233 MOLECULAR COMPOSITION, FLUIDITY OF MEMBRANES AND FUNCTIONAL PROPERTIES OF HUMAN LIVER MITOCHONDRIA AND MICROSOMES. Gheorghe Benga, Department of Cell Biology, Faculty of Medicine, Medical and Pharmaceutical Institute Cluj-Napoca, 6 Pasteur St., 3400 Cluj-Napoca, Romania.

Membranes of human liver mitochondria and microsomes exhibit some peculiarities of lipid and protein composition compared to their counterpart in the rat (1-3). These include: a) an increased lipid content, human liver subcellular membranes containing twice as much lipid as those from the rat of each of the lipid classes; b) differences in essential fatty acid composition of total lipids and phospholipids: human liver membranes contain more linoleic acid and less arachidonic acid than those of the rat; c) an increased content of hydrophobic aminoacids in human membranes; d) although the unsaturation of lipids is lower in human than in rat liver membranes a higher fluidity of human membranes has been inferred from spin label studies. This might arise from a lesser immobilization of lipids by proteins in comparison with the rat liver membranes. This could explain the fragility of human liver organelles (4), as well as certain pecularities of their enzymic activities (5-7). The implications of these findings in liver pathology will be discussed.

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234 COVALENTLY-CROSSLINKED MATRICES IN MUSCLE, NERVE AND OTHER CELLS. Ariel G. Loewy, Dolores E. Shupp-Byrne, Cheryl A. Storm. Department of Biology, Haverford College, Haverford, PA, 19041 USA. When chicken pectoral muscle is extracted exhaustively with 6M guanidine HCl, 1% Triton X100, 5% 2-mercaptoethanol, pH8.5 a "tissue ghost" remains containing 1% of the original protein. Scanning EM images show muscle "fiber ghosts" embedded in an extracellular matrix. Treatment of tissue ghosts with high purity collagenase releases the fiber ghosts consisting of less than 0.2% of the original protein. Fiber ghosts resemble muscle fibers in shape and retain striations and indentations left by the extracted nuclei. Transmission microscopy of fiber ghosts show dense bands (interpreted as Z-discs associated with insoluble matrix) interconnected by superthin filaments. The amino acid content of the matrix is high in hydrophobic residues. Fiber ghosts dissociate in 5% SDS or 60% formic acid into a very large polymerized matrix which does not enter a 2.5% acrylamide gel. Antibodies raised against tissue ghosts cross-react with titin and myosin rod protein and with glycerol-extracted myofibrils giving a pattern of bands in immunofluorescence similar to that obtained with anti-titin antibody. Anti-fiber ghost antibodies cross-react with the extracted ghosts of gizzard and nerve cells. Extraction and collagenase treatment of gizzard leaves cell ghost which remain interconnected, suggesting the presence of covalently-crosslinked transcellular connections. Similar treatment of nerve axons leave ghosts which in transmission microscopy show vesicular profiles, fiber bundles and superthin filaments. Prelminary measurements show that the insolubable cytomatrix contains N^{ϵ}-(γ-glutamic)lysine bands. Work is in progress to dissect the matrix by cleaving enzymatically glu-Lys bonds and identifying the constituent protein chains of the cytomatrix. (Supported by NIH Grant Am 34503 and NSF Grant DCB 851112).)

235 CORNIFIED ENVELOPE COMPETENCE AND SUBCELLULAR DISTRIBUTION OF TRANSGLUTAMINASE ACTIVITIES IN CULTURED HUMAN CELLS.

Rainer Schmidt, Serge Michel, Uwe Reichert. Centre International de Recherches Dermatologiques, Sophia Antipolis, F-06565 Valbonne Cédex, France.

Striking differences in transglutaminase (TG) activities are observed in the subcellular fractions (cytosol, intracellular particulate, purified plasma membranes) derived from different human cells in culture (normal human keratinocytes, transformed human keratinocyte line SV-K14, hepatoma cell line PLC-PFR5). All of these cells attain under defined conditions a certain competence to synthesize a cornified envelope. This competence is directly correlated with the specific activity of the plasma membrane bound form of TG (TGm). SV-K14 cells gain 100% envelope competence after 4 days of serum starvation. During this period a decrease of cytosolic TG (TGc) activity and an increase of TGm activity is observed which correlates with the increase in envelope competence. Qualitatively the same holds true for the hepatoma cells, however, with the difference that the total TGm activity is much lower and thus their envelope competence. Retinoids inhibit the development of envelope competence by suppressing the normally observed increase in TGm activity. The TGm of SV-K14 cells is distinct from their TGc since only TGc is decorated in the immunoblot by a polyclonal antibody raised against guinea pig liver TG. Normal human keratinocytes, when compared with competent SV-K14 cells, express a very low TGc activity, but an equivalent TGm activity. In these cells, neither of the two TG forms is decorated by the above mentioned antibody. These findings can be explained by the assumption that two genetically different TG enzymes are cell-type specifically expressed, one of which is, under certain conditions, able to be inserted into the plasma membrane where it catalyses the formation of a cornified envelope.

236 INVOLUCRIN: A MULTISITE SUBSTRATE OF THE MEMBRANE ASSOCIATED EPIDERMAL TRANSGLUTAMINASE.

Marcia Simon and Howard Green. Department of Physiology and Biophysics, Harvard Medical School, Boston, Mass. U.S.A. 02115.

A transglutaminase-catalyzed crosslinking process characteristic of keratinocytes leads to the formation of the insoluble corneocyte envelope. The essentials of this process take place in vitro in a reconstituted system derived from subcellular fractions. A particulate fraction containing membrane bound envelope precursor proteins and the keratinocyte specific transglutaminase is combined with cytosolic proteins; when the enzyme is activated by Ca^{++}, cytosolic proteins are removed from solution and crosslinked to particulate proteins. Involucrin, a cytosolic protein known to be the precursor of the envelope, is more efficiently crosslinked than other cytosolic proteins. The sequence of involucrin is such that many of the peptide fragments generated by tryptic hydrolysis are carboxamide donors in the transglutaminase reaction. These multiple carboxamide donor sites span over half of the protein and are likely to account for the ability of involucrin to promote the crosslinking of proteins of the particulate fraction (membrane proteins).

237 THE INTERNAL THIOLESTER IN α_2MACROGLOBULIN AND IN COMPLEMENT COMPONENTS C3 AND C4.

Fred VAN LEUVEN, Center for Human Genetics, University of Leuven, Campus Gasthuisberg, O&N6, Herestraat, B-3000 Leuven, BELGIUM.

The endopeptidase inhibitor $\alpha_2$macroglobulin (α_2M) shares with pregnancy zone protein (PZP) and with complement components C3 and C4 the unique sequence Gly-Cys-Gly-Glu-Gln. In the native proteins this allows for the construction of a thiolester linkage : a covalent bond between the cysteine sulfhydryl group and the δ-carboxyl group of the glutamine. This configuration becomes very reactive upon activation by proteolytic cleavage of the polypeptide chain. The resultant depends on the protein : complement components C3 and C4 form covalent linkages (either amides or esters) at cell-surfaces, α_2M and PZP from covalent linkages with the endopeptidase. The necessity for these linkages to form, seems clear in the function of C3 and C4 since they are part of an amplification mechanism in the complement cascade at the surface of the target-cell. On the other hand in the effective functioning of α_2M, the covalent linkage of the proteinase to the α_2M-chains is not essential to the stability of the resulting α_2M-proteinase complex. Treatment of α_2M with methylamine leads to derivatisation to the δ-methylamide of glutamine and results in a conformational change of α_2M, also seen when proteases are bound. Therefore, in α_2M the internal thiolesters main function seems to procure the active state, both in terms of conformation and in terms of expression of the receptor-recognition site.
α_2M, isolated from species other than human, do not change conformation when treated with methylamine. Also, in conditions where the liberated sulfhydryl-group is cyanylated, human α_2M does not change its conformation when treated with methylamine. In both instances, the inactivated proteins are recognized by the cellular receptor. Ultimately then, the internal thiolester seems only essential for expression of the receptor-recognition site.

238 RETINOIC ACID - INDUCED EXPRESSION OF TISSUE TRANSGLUTAMINASE IN MOUSE PERITONEAL MACROPHAGES. Peter J. A. Davies. Department of Pharmacology, University of Texas School of Medicine at Houston, Houston, Texas, U.S.A. 77096.

Transglutaminases are enzymes that catalyze the covalent cross-linking of proteins. Tissue transglutaminase is a specific intracellular transglutaminase that can be induced to high levels (1% of cellular protein) in resident mouse peritoneal macrophages exposed to serum-containing media. We have identified the transglutaminase-inducing activity of serum as trans-retinoic acid (tRA) bound to the serum retinol binding protein. Nanomolar levels of tRA induce acute and specific increases in tissue transglutaminase gene expression within minutes of addition to mouse macrophages. Analogues of cyclic AMP and agents that elevate intracellular cyclic AMP synergistically potentiate the induction of tissue transglutaminase and phorbol esters and pertussis toxin both inhibit the induction. In addition to the induction of tissue transglutaminase, retinoids also increase the levels of transglutaminase activity detectable on the surface of macrophages. The molecular identity of this cell surface transglutaminase remains obscure. Induction of this activity is associated with the covalent cross-linking of cell surface proteins, particularly fibronectin. The induction of tissue transglutaminase and a cell surface transglutaminase activity and the cross-linking of cell surface fibronectin are also features of macrophages activated in vivo. Our findings suggest that large accumulations of both intracellular and extracellular transglutaminase activity and the covalent cross-linking of cell surface proteins may be impotant components of macrophage activation.

239 PARTICIPATION OF TRANSGLUTAMINASE IN MOTILE EVENTS ASSOCIATED WITH PROINSULIN CONVERSION AND INSULIN RELEASE IN THE PANCREATIC B-CELL. Willy J. Malaisse and Ramon Gomis. Laboratory of Experimental Medicine, Brussels Free University, Brussels, Belgium

Both the conversion of proinsulin to insulin and the release of the latter hormone involve the oriented transiocation of microvesicles and secretory granules within the pancreatic B-cell. Ultrastructural, biochemical, functional and cinematographic observations have documented the role of the microtubular-microfilamentous system in such intracellular movements. Transglutaminase, which catalyzes the cross-linking of proteins, could conceivably participate in these motile events. Transglutaminase activity in islet homogenates is activated by Ca^{2+}, and D-glucose increases cytosolic Ca^{2+} activity in the B-cell. Several inhibitors of transglutaminase activity in islet homogenates were examined for their effect upon functional events in intact islets. The most reliable information was obtained with glycine methylester which decreased [^{14}C]methylamine incorporation into endogenous proteins of intact islets, caused a rapid and reversible inhibition of insulin release, retarded the onset and increased the half-life time of proinsulin conversion. Furthermore, [2,5-^{3}H]histamine is incorporated into endogenous TCA-precipitable material whether in islet homogenates or intact islets. The latter incorporation represents a temperature-sensitive and time-related process stimulated by D-glucose in the presence, but not in the absence, of extracellular Ca^{2+}. Thus, D-glucose apparently causes a Ca^{2+}-dependent activation of transglutaminase in intact islet cells. The activity of the enzyme may also be affected by the changes in cytosolic redox state induced by D-glucose. This view is supported by the dramatic inhibition of transglutaminase in homogenates prepared from islets first exposed to 2-cyclohexene-1-one. Further work is required to identify the endogenous protein substrate(s) for transglutaminase in the islet cells.

240 TRANSGLUTAMINASE ACTIVATION DURING CELL STIMULATION. THE MAST CELL EXAMPLE. Laszlo Fesus. Department of Biophysics, University School of Medicine, Debrecen, Hungary, H-4012.

Several morphological and biochemical data, which show that a significant portion of tissue transglutaminase is localized in the plasma membrane of various types of cells and tissues, have been accumulated recently. It seems that this "cryptic" form of the Ca^{2+}-dependent transglutaminases is released from the lipid environment and activated during cell stimulation. A clear example of this phenomenon is the stimulation of mast cells. The identification of transglutaminase in the mouse mast cell line PT18 was accomplished through its characteristic catalytic properties (specificity, calcium dependency, and inhibition by iodoacetamide); and by both immunoprecipitation and Western blot analysis using affinity purified antibody. A portion of the enzyme is associated with the membrane fraction. The enzyme activity in these cells is increased in association with the release of histamine induced by an IgE-dependent mechanism or by exposure to the ionophores A23187 or Br-x537A. The increase in transglutaminase activity was paralleled by the presence of more enzyme in the cytosol and a marked increase in the level of protein-bound gamma-glutamylhistamine. The latter was determined in radiolabeled form in mast cells that were either metabolically labeled with ^{3}H histidine or incubated with ^{3}H histamine before degranulation. The highest level of bound gamma-glutamylhistamine was found in the immunologically stimulated cells. Enzymatic activity and the gamma-glutamyl derivative were associated primarily with the cells, both before and after stimulation. The protein fraction containing gammaglutamylhistamine is a high molecular weight polimer crosslinked either before or during cell stimulation.

241 LOCALIZATION OF COVALENTLY BOUND [^{3}H]PUTRESCINE IN THE CHO INSOLUBLE CROSS-LINKED MATRIX AFTER EXPOSURE TO SODIUM BUTYRATE. Simone Beninati and Mauro Piacentini. Department of Biology, 2nd University of Rome (Tor Vergata), Via O.Raimondo, 00173 Rome (Italy) and National Institute of Dental Research, National Institutes of Health, Bethesda, Md 20205 (U.S.A.).

CHO (chinese hamster ovary) cells grown in the presence of [^{3}H]putrescine, after sequential exposure to sodium butyrate, DFMO (difluoromethyl ornithine) and MGBG (methylglyoxal bis (guanylhydrazone)) respectively, were shown to have increased transglutaminase activity and reduced spermidine synthesis, associated with recoverable TCA-insoluble radiolabeled amine. The proteolytic digestion of the CHO acid-insoluble material revealed the presence of N^1,N^4 bis-(γ-glutamyl)putrescine, N^1-(γ-glutamyl)putrescine and hypusine. The bis-glutamyl putrescine derivative is indicative of covalent cross-linkages between the diamine and proteins. Glycerol extraction of CHO cells led to the recovery of radioactive label in the form of hypusine in the soluble fraction and in the form of covalently bound [^{3}H]putrescine in the insoluble fraction. The glycerol-insoluble fraction is thought to contain the cytoskeleton with the related membrane components and it is here that Glutamine-Lysine cross-links probably occur (A.G.Loewy and S.S.Matacic, Biochim.Biophys.Acta 668, 167-176, 1981).

Preliminary studies on the characterization of [^{3}H]putrescine-protein complexes showed that the labeled diamine is distributed in protein aggregates of molecular weights greater than 500,000 daltons and in a peptide of about 70,000 daltons.

242 **IDENTIFICATION OF MEMBRANE PROTEINS INVOLVED IN THE WATER PERMEABILITY OF HUMAN ERYTHROCYTES.** **Gh.Benga (1), O.Popescu (1), V.I.Pop (1), Victoria Borza (2), Ildiko Mocsy (3), Ana Muresan (1). (1) Department of Cell Biology and (2) Biochemistry, Medical and Pharmaceutical Institute Cluj-Napoca and (3) Institute of Hygiene and Public Health, Cluj-Napoca, Romania.**

We have previously studied through nuclear magnetic resonance some effects of sulfhydryl reactive (SH) reagents on water diffusion across human erythrocytes. We have extended our studies to follow the time course and concentration dependence of inhibition induced by mercurials on erythrocytes and resealed ghosts' water permeability, preceded or not by preincubation with N-ethylmaleimide (NEM), a non-inhibitory SH reagent. The treatment with NEM prior to exposure to p-chloromercuribenzene sulfonate (PCMBS) results in the inhibition of water exchange occuring faster and at a lower concentration of mercurial. The inhibition induced by mercurials was correlated with binding of ^{203}Hg-PCMBS to erythrocyte membrane proteins. In varying PCMBS concentration, temperature and time of incubation, with both erythrocytes and ghosts, the inhibition was maximal with a binding of ~10 nmole/mg protein. We found by polyacrylamide gel electrophoresis that binding to band 3 and the polypeptides in band 4.5 occur under these conditions, with approximately 1 mole of mercurial bound per mole of protein. This suggests that proteins in both of these bands are associated with water channels in erythrocytes. Inhibition of water transport by sulfhydryl reagents does not induce major morphological changes in the cells as assessed by freeze-fracture and scanning electron microscopy.

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243 *FLOW CYTOMETRIC QUALIFICATION OF PROSTAGLANDIN $F_{2\alpha}$ TREATED BOAR SEMEN AFTER LONG TERM STORAGE*

T. Takács(1), J. Matkó (2), L. Mátyus (2), R. Gáspár (2) S. Damjanovich(2), (1) Animal Breeding Enterprise, H-4026 Debrecen, (2) Department of Biophysic, University Medical School of Debrecen, H-4012 Debrecen.

Prostaglandin are known as very efficient promoting factors in male and female reproductive processes. The prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$) and its analogues are widely used in the veterinary practice for improvement the reproductive performance of domestic animals.

Several authors have investigated the effect of $PGF_{2\alpha}$ supplementation on boar semen. In most cases a considerable improving effect on the sows fertility rate was observed when using semen treated with $PGF_{2\alpha}$. However the in vitro viability of spermatozoa seemed to decrease during storage after the treatment with pharmacological doses of $PGF_{2\alpha}$ as determined microscopically on the base of the proportion of motile cells.

A flow cytometric method was introduced in our laboratory to determine the viability of spermatozoa. Reliable staining methods, using different fluorescent dyes like 3,3'-dihexiloxacarbocyanine iodide (DiOC$_6$(3)) and propidium iodide (PI), were applied to detect sperm viability. A modified Becton-Dickinson FACS III flow cytometer was used to measure the fluorescence intensity of individual cells in large sperm populations.

Both treated and untreated cells showed a slight deterioration during a long term (96^h) storage at 17°C. A heat stress test at 42°C showed a significant decrease in the viability of both groups. No significant difference could be observed, however, between the viability indices of $PGF_{2\alpha}$-treated and untreated samples, in any cases.

On the other hand a significant difference was observed in the values of viability indices determined by the microscopic and flow cytometric methods, respectively. Possible reasons of these contradictory values will also be discussed.

244 RECEPTOR-MEDIATED ENDOCYTOSIS AND INTRACELLULAR ROUTING OF RICIN AND RICIN CONJUGATES IN CULTURED BREAST EPITHELIAL CELLS.

Bo van Deurs and Ole William Petersen, Department of Anatomy, The Panum Institute, Blegdamsvej 3, DK 2200 Copenhagen N, Denmark.

Ricin, a representative of a group of extremely toxic plant and bacterial polypeptides, consists of two chains, each of about 30 kD. The A-chain is an enzyme which, following translocation into the cytosol, interferes with the ribosomal 60S subunit. The B-chain is a lectin binding to cell surface receptors, that is, galactose-terminating glycoproteins and -lipids. Ricin is functionally monovalent, since each molecule binds to only one receptor site (1,2).

Using ultrastructural immunocytochemistry we have shown that ricin is taken up in cultured cells by receptor-mediated endocytosis (RME) via coated pits and vesicles. Binding of ricin to receptors at 4°C can be prevented with preincubation in 0.1 M lactose. Within the cell, ricin reached the vacuolar and tubulovesicular portions of the endosomal system (VP and TVP, respectively), and after 30 to 60 min also trans-Golgi elements and lysosomes (2). Identical results have been obtained with a monovalent ricin-HRP conjugate (Ri-HRP; prepared by the SPDP method, followed by gel filtration and SDS-PAGE), and this conjugate was almost as toxic as native ricin (measured as inhibition of cellular incorporation of ^{3}H-leucine). In contrast, polyvalent Ri-HRP and ricin-colloidal gold (Ri-Au), being internalized in the same way as native ricin and monovalent Ri-HRP, mainly reached the VP and lysosomes. The TVP was reached to a smaller degree, and Golgi elements were never labeled by the polyvalent conjugates (2,3). Both polyvalent Ri-HRP and Ri-Au were considerably less toxic than monovalent Ri-HRP and native ricin.

The fact that native ricin and all conjugates reached the endosomal system (especially the VP), whereas only the monovalent conjugate and native ricin were thereafter directed to the Golgi complex, suggests that sorting, mediated by the valency of ligands and/or ligand-receptor complexes, takes place in endosomes. This is in agreement with previous biochemical findings on the Fc-receptor and the LDL receptor (4-6).

Since optimal ricin A-chain translocation apparently requires a neutral pH (2), and since endosomes, lysosomes and trans-Golgi elements are acidic, whereas the tubulovesicular portion of the endosomal system at least in part may have a higher pH (7-10), our observations suggest that the TVP is a likely site for ricin A-chain translocation. Moreover, internalized ricin and/or ricin receptors are in part recycled to the cell surface, and this recycling presumably takes place from the TVP and/or from trans-Golgi elements. It is concluded, however, that the TVP and the trans-Golgi elements are two distinct compartments for the following reasons: (a) while Ri-HRP reaches trans-Golgi elements in two breast cancer cell lines (MCF-7 and T47D), this was not seen in primary cultures of normal breast epithelium; (b) serial section analysis revealed in all three cell types Ri-HRP in a complex reticular, tubulo-vesicular system, often connected with the vacuolar portion of the endosomal system (11).

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245 DISSECTION OF ENDOSOMAL COMPARTMENTS IN RAT HEPATOCYTES. Pierre J. COURTOY, Laboratory of Physiological Chemistry, University of Louvain Medical School and the International Institute of Cellular and Molecular Pathology, 75 avenue Hippocrate, B-1200 Brussels, Belgium.

A large variety of ligands are picked up by rat hepatocytes at their sinusoidal surface by receptor-mediated endocytosis (1), and subsequently dispatched into three major intracellular pathways. In normal conditions, most ligands (including galactose-exposing bovine serumalbumin, galBSA), but only few receptors, are discharged into lysosomes and digested (degradative pathway). Most receptors and some of those ligands which remain membrane-associated despite exposure to the acidic pH of endosomes, apparently by-pass lysosomes and are returned to the sinusoidal surface (recycling pathway). It is generally accepted that acidity in endosomes generates a phase partition, and that tubulo-spherical structures further segregate the fluid phase into large vesicles destined to lysosomes, from the membrane phase, which is retrieved to the cell surface by narrow tubules or vesicles. This is in keeping with the evidence that only a small fraction of internalized plasma membrane constituents is transferred to lysosomes at every endocytic cycle (see Draye et al., and Moguilevsky et al., this meeting). In the case of hepatocytes, cell polarization defines a third pathway, whereby membrane constituents are transferred across the cell and excreted into bile (transcytosis). In the rat, the transcellular pathway can be demonstrated by J chain-containing polymeric IgA (pIgA). The distinct pathway of pIgA is explained by its unique ability to establish one or several disulfide bridges with the ectoplasmic domain of its receptor (an intrinsic membrane protein, called secretory component, SC). This interaction may be promoted by a disulfide-interchange enzyme located along the vacuolar apparatus (3). During, or at the end of the transcellular transfer, pIgA-SC is cleaved off the membrane by an as yet unknown endopeptidase, and excreted into bile with its bound ligand as secretory IgA.

We have compared the endosomal compartments involved in the degradative and transcytotic pathways, using galBSA and pIgA conjugated with horseradish peroxidase (HRP). Such conjugates make it possible to compare in the same liver (a) the ultrastructural aspect, topography and stereological parameters of ligand-containing organelles in the fixed tissue, (b) the subcellular distribution of the ligand in the homogenate and (c), the membrane composition of selected endosomal populations, which have been further purified by a ligand-specific density shift procedure.

By electron microscopy, after peroxidase cytochemistry (4), galBSA-HRP and pIgA-HRP were both internalized through coated pits into peripheral endosomes, which frequently displayed tubulo-spherical profiles (1-5 min). Thereafter, both ligands were translocated within 5-20 min to the pericanalicular region, where galBSA-HRP occurred mostly in large structures, including lipoprotein-filled vesicles and multivesicular bodies, whereas pIgA-HRP was mostly found in numerous small tubules or vesicles. After 30 min, galBSA-HRP was concentrated in lysosomes, while pIgA-HRP-containing structures started fusing with the canalicular membrane, and secretory IgA was excreted. Neither ligand was detected in Golgi stacks. At 10-20 min, for each of the two endosomal populations, contribution to the hepatocyte volume was < 1 %, and the membrane area corresponded roughly to one-third of the pericellular membrane.

By cell fractionation, galBSA and pIgA (or their peroxydase conjugates) were associated at 3 min with small particles (pelleted in the microsomal fraction, i.e. between 0.25 and 3 10^6 g x min), and of low equilibrium density (~1.13 g/ml in sucrose gradients). At 10 min, galBSA peaked in larger structures (recovered in the L fraction, i.e. between 0.03 and 0.25 10^6 g x min) and even lower equilibrium densities (~1.11). These two distributions were clearly distinct from the bulk of plasma membrane marker enzymes (5'-nucleotidase, alkaline phosphodiesterase I, and Mg^{++}-ATPase). After 20-30 min, galBSA distribution closely coincided with that of cathepsin B or N-acetyl-ß-glucosaminidase (two lysosomal markers). Chloroquine accumulation apparently delayed galBSA transfer along these successive host compartments. In contrast, the distribution of pIgA remained essentialy identical thoughout its transepithelial transfer (P fraction; equilibrium density : ~1.13).

Preparations enriched in endosomes were further purified by a density-shift procedure based on the selective accumulation of HRP-reaction product in the isolated, unfixed ligand-containing particles. This procedure is specific and allows to test for the association or dissociation of constituents which co-distribute in a conventional fractionation system (5). When galBSA-HRP and pIgA were injected simultaneously, both ligands were mostly recovered at low densities from 5 to 20 min. The density shift of galBSA-HRP-containing structures induced a concomitant displacement of most of pIgA at 5 min, but this proportion declined at 10 and further at 20 min. This confirms that galBSA and pIgA were co-internalized into identical endosomes, where they were sorted and segregated into distinct endosomal populations. The membrane composition of these populations was calculated from the proportion of marker enzymes that was shifted concomitantly with either galBSA-HRP or pIgA-HRP, taking the yield into account (6). For either ligands, host endosomes always contained less than 4 % of N-acetyl-ß-glucosaminidase of the homogenate. Endosomes carrying galBSA-HRP from 5 to 30 min contributed about 1.6 % of 5'-nucleotidase and 2.7 % of alkaline phosphodiesterase I. For pIgA-HRP, corresponding values were 1.4 and 2.3 % at 10 min, but reached 13 and 19 % at 20 min. This major change in the composition of pIgA-containing endosomes ("maturation") is reminiscent of the reported concentration of 5'-nucleotidase and alkaline phosphodiesterase I at the bile front (7), and may indicate that pIgA is transferred in the pericanalicular region to vesicles competent to fuse with the bile canalicular membrane.

In conclusion, galBSA and pIgA are co-internalized into common "peripheral" endosomes, and subsequently segregated into distinct "central" endosomes, respectively committed to the degradative and the transcytotic pathways. Both endosomal populations differ in size, content and membrane composition. The latter could include distinct recognition signals for the differential addressing of endosomes.

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246 RECEPTOR DOMAINS IN THE PLASMA MEMBRANE OF CULTURED CELLS.

Horst Robenek. Lehrstuhl für Medizinische Cytobiologie, Domagkstr. 3, Universität Münster, 4400 Münster, FRG

The function of the plasma membrane to respond to changes in the extracellular environment depends upon the presence of receptors on the outer cell surface. Over the past few years, important advances in the understanding of the biology of receptors have been made.
Biochemical methods have been the mainstay for the assessment of the presence of receptors in a cell population. However, averaging biochemical techniques and existing methods for the visualization of receptors lack the resolution for the visualization of receptors within the plasma membrane of a single cell.
With the aid of the platinum-carbon replication technique combined with the unique advantages of colloidal gold labeled ligands and antibodies - a method for high resolution electron microscopic visualization of receptors - we demonstrate the existence of characteristic microdomains in the distribution pattern of receptors in the plasma membrane of cultured cells. Here, the distribution of receptors for Low Density Lipoprotein (LDL) in human skin fibroblasts, acetylated LDL, High Density Lipoprotein (HDL), ironsaturated transferrin and Bovine Serum Albumin (BSA) in mouse peritoneal macrophages and Immunoglobulin A (IgA) in primary rat hepatocytes was mapped.
When fibroblasts were incubated at 4°C for 1h with LDL-gold complexes, the gold particles were visualized almost exclusively in coated pit regions. Exposure of macrophages to acetylated LDL-gold conjugates at 4°C for 1h results in preferential binding in the intermediate region of the plasma membrane surface. The gold particles are more or less randomly distributed, indicating that the distribution of unoccupied acetyl-LDL binding sites in the native membrane is diffuse.
The distribution pattern of HDL-gold conjugates resembles that of acetyl-LDL-gold conjugates. However, the HDL-gold conjugates tend to aggregate in clusters of irregular outline.
After incubation of macrophages with gold-labeled transferrin most of the transferrin-gold conjugates were found in the peripheral region and, to a lesser extent, in the central region of the plasma membrane. BSA-gold conjugates are distributed more or less randomly in a small amount over the entire plasma membrane of a macrophage.
The finding that a special type of receptor is present in very high densities on a differentiated membrane area (while being absent on other areas) suggests that the cell may have some sort of control over the topographical distribution of its receptors. Because the plasma membranes are expected to have common fluid bilayers, it is of interest to identify the means by which the cells maintain the existence of these receptor domains and prevent the randomization of their receptors. At present the exact nature of the control and its effectors is completely unknown. However, most probably cytoskeletal elements such as microtubules and microfilaments are involved.
To study possible interactions of cytoskeletal elements with the plasma membrane receptors, the influence of cytochalasin B on the topography and dynamics of IgA-secretory component complexes (IgA-SC) on the surface of cultured hepatocytes was investigated. The distribution of IgA-gold conjugates after incubation at 4°C was similar in normal and cytochalasin B treated hepatocytes and was characterized by a mixed random type, intermediary to the extremes of random scattering on one side and exclusive clustering on the other side. Since direct decoration of SC by anti-SC-antibodies or by gold-labeled IgA subsequent to fixation led to virtually the same results, this distribution seems to reflect that of the unoccupied receptor. After raising the temperature to 37°C redistribution of particles and their gradual uptake into coated vesicles was observed in control cultures. In contrast huge patches of IgA-gold conjugates were formed at the cell periphery of cytochalasin B-treated hepatocytes within 20min at 37°C, while central regions were cleared.
This patch formation was triggered by binding of both, unlabeled or labeled IgA, but could not be observed with the unoccupied receptor as demonstrated by gold-labeled antibodies against secretory component. These results demonstrate that the clustering of IgA-receptors is ligand-induced and a well controlled process with respect to cluster size and topography. The observed impairment of this control by cytochalasin B which results in patch formation extends and specifies previous hypotheses that microfilaments play a role in the anchorage of receptors and their mobility in the plasma membrane.
Although the high resolution technique applied in this study provides some new information on the possible interactions between ligand-receptor-complexes and microfilaments, they are unfortunately indirect. Therefore at present work in our laboratory is focussed on the development of new approaches which make it possible to study the internal cytoplasmic surface of cultured cells to get more direct information on the association of cytoskeletal elements with plasma membrane components, i. e. receptors. Our preliminary results show that the lysis squirting technique can be combined with immuno-gold labeling of various structural components associated with the cytoplasmic surface of the plasma membrane. Subsequent processing of the specimens for platinum-carbon replicas permit an unambiguous identification and locatization of molecules in the replica. As an example, we show here the labeling of microfilaments and coated structures with specific antibodies in combination with either gold labeled second antibodies or protein A - gold.

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247 COLCHICINE-INDUCED TUBULAR, VESICULAR AND CISTERNAL ORGANELLES - PHOSPHATASE CYTOCHEMISTRY AND ENDOCYTOSIS STUDIES

Adolf Ellinger and Margit Pavelka, Institute of Micromorphology and Electronmicroscopy, Schwarzspanierstrasse 17, 1090 Vienna, Austria

Administration of the antimicrotubular agent colchicine to adult rats (0.5mg/100g of body weight for 6hr) induces formation of extended aggregates of tubular, vesicular and cisternal organelles in the absorptive cells of the small intestine. Most of the clustered organelles showed intense staining for thiamine pyrophosphatase, acid phosphatase and acid trimetaphosphatase (1). Endocytosis studies, using horseradish peroxidase (HRP), showed that the colchicine-induced clustered organelles are recipients for molecules taken up at the apical and basolateral cell surface. Once internalized, HRP was rapidly sequestered to the clustered tubules/vesicles, to multivesiculated bodies and dense bodies. At 60min after HRP-administration all of the clustered tubules/vesicles contained HRP-reaction product.

The morphological appearance of the aggregated tubules/vesicles in close vicinity to multivesiculated vacuoles reminds of receptosome (2) / CURL (3) elements (compartment of uncoupling receptor and ligand) and suggests that at least some of the clustered organelles belong to this system. On the other hand, the phosphatase reaction patterns point to the lysosomal nature of these tubules, vesicles and cisterns.

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248 ROLE OF MULTIVESICULAR ENDOSOMES IN LIGAND RECYCLING

J.Timár(1),K.Lapis(1),D.McIntosh(2),N.Bradley(2),A.J.S.Davies(2).(1) Ist Inst. of Path.and Exptl.Canc.Res.,Semmelweis Med.Univ.Budapest,Üllői u.26.1085-Hungary, (2)Biology and Surgery Sect.Chester Beatty Labs.,Inst.Canc. Res.London,U.K.

The multivesicular endosomes /bodies/ belong to a quite controversial system, to the so-called intracellular acidic compartment. It was recently demonstrated their central role in transferrin-receptor recycling/Pau et al.1985/.We have studied their role in the traffic of gal-specific toxic lectin -Ricin and Ricin-A chain-based immunotoxin in human cancer cells EJ by immunoelectronmicroscopy /Timár et al.1985/. Ligands were labeled after binding to cell surface with antibody-coll.gold complex. Ricin was quickly internalised into intracellular acidic compartment - dominantly into multivesicular endosomes /MVES/.Parallelly with the internalisation Ricin was recycled back to the cell surface on vesicles derived from MVES. The NEM treatment after 3o min. Ricin internalisation can block the lysosomal uptake of the ligand, while stimulates the recycling on vesicles. A monoclonal antibody-targeted Ricin - the FIB75-Ricin A immunotoxin - internalised according to the pattern of the monoclonal antibody-receptor and accumulated in lysosomes. Using Ricin B-chains to reconstitute the holotoxin on the monoclonal receptor, it was possible to alter the traffic of immunotoxin, to bypass the lysosomes and to accumulate in MVES. The presence of the complex in MVES resulted in a continuous recycling to the cell surface via membrane vesicles. The chloroquin inhibition of lysosomal acidification altered the intracellular traffic of immunotoxin in a similar way. These results provided evidences for the central role of MVES in receptor recycling.

249 ENDOSOME-LYSOSOME FUSION IS INHIBITED BY CHLOROQUINE AND MONENSIN.

Kai Stenseth, Ulf Hedin and Johan Thyberg, Department of Histology, Karolinska Institutet, S-104 01 Stockholm, Sweden.

Endocytosis is the principal mechanism for cellular uptake of macromolecules. By inward budding of the plasma membrane, exogenous material is internalized into small peripheral vesicles, which rapidly fuse with each other to form larger endosomes. Although occasional exceptions are known, endosomes normally empty their content into lysosomes for subsequent degradation by acid hydrolases. Little is known about how this intracellular routing of endocytosed material is regulated. It is, however, interesting to note that both endosomes and lysosomes have been found to have a low intraluminal pH. To study the possible role of pH in the fusion between these organelles, we examined the effect of the weak base chloroquine and the Na^+/H^+-ionophore monensin on the uptake of ferritin in cultured murine peritoneal macrophages. Lysosomes were preloaded with horseradish peroxidase (HRP) and after a 45-min chase in HRP-free medium, the cells were exposed to ferritin in control medium or medium containing 50 µM Chloroquine or 5 µM monensin. They were then fixed and processed for electron microscopy and cytochemical demonstration of peroxidase activity. In control cells, the ferritin particles were rapidly ingested and transferred into HRP-labeled lysosomes. Already after 30 min the majority of these also contained ferritin, the amount of which thereafter steadily increased. In contrast, lysosomal transfer of ferritin was strongly inhibited in monensin- and, to a slightly lower degree, chloroquine-treated cells. Although the rate of endocytosis was somewhat lowered by the drugs, uptake via coated and uncoated vesicles continued throughout the observation period of 120 min. Thus, ferritin accumulated in endocytic vesicles and endosomes unable to fuse with HRP-labeled lysosomes. Since both chloroquine and monensin, though acting by different mechanisms, have been found to rapidly raise the pH of acid intracellular compartments, our results suggest that endosome-lysosome fusion is a pH-dependent process.

250 THE FATE OF INSULIN INTERNALIZED BY RAT ADIPOCYTES. David G. Fernig and R. John Mayer, Dept. of Biochemistry, University of Nottingham, University Hospital, Clifton Boulevard, Nottingham NG7 2UH, U.K.

[^{125}I]insulin internalized by rat adipocytes can be isolated in an endosomal compartment by fractionation of adipocyte homogenates on sucrose density gradients (Suzuki and Kono, 1979; Jeffrey et al. (1985). This endosomal compartment has been further characterized using a combination of differential centrifugation, a modified version of the sucrose gradient described by Jeffrey et al. (1985), and a 16%-50% nycodenz gradient. Nycodenz gradient fractions containing [^{125}I]insulin do not co-distribute with either lysosomal (acid phosphatase, B-D glucuronidase, B-hexoseaminidase) or other subcellular organelle marker enzymes. In pulse chase experiments [^{125}I]insulin was rapidly lost from the adipocyte cell interior during the 30 min chase in Medium 199 in the presence of a 1000 fold excess of insulin. Only 35% of this loss is accounted for by degradation: 65% of [^{125}I]lost from the adipocytes is recovered intact in the culture medium as judged by trichloroacetic acid precipitatability.

Jeffrey, I.W., Flint, D.J., Vernon, R.G., Mayer, R.J., FEBS Letts. (1985) 182, 451-454.
Suzuki, K. and Kono, T. (1979) J. Biol. Chem. 254, 9786-9794.

251 IMMUNO-ISOLATION OF A PLASMA MEMBRANE AND AN ENDOSOMAL FRACTION FROM BHK CELLS: ANALYSIS WITH CELL-FREE FUNCTIONAL RECONSTITUTION STUDIES. J. Gruenberg and K.E. Howell, European Molecular Biology Laboratory, Postfach 10.2209, 6900 Heidelberg, FRG.

We have designed a new approach for the immuno-isolation and subsequent characterization of the compartments of the endocytic pathway. The membrane-spanning G protein of VSV is implanted in the plasma membrane of BHK cells at low pH in its native trans-bilayer conformation (Gruenberg and Howell, Eur. J. Cell Biol. 38,312). The exoplasmic domain of G is used as antigen for plasma membrane immuno-isolation. When cells with implanted G are incubated at 20°C for 2 hrs (or 37°C, 20 min), G clears from the cell surface and is internalized in endosomes where it colocalizes with HRP (a fluid phase marker). The G cytoplasmic domain, exposed on the surface of endosomal elements, is used as antigen for the endosome immuno-isolation. The yield from the homogenate is 10-20% for both fractions with an enrichment of 15x over controls. The iodinated protein patterns of both fractions were studied following in situ iodination of the cellular compartments. Three major populations are found: proteins common to both compartments, enriched in the plasma membrane or in endosomal fractions. All proteins iodinated at the cell surface were internalized and recovered in the endosomal fraction.

The endosomal fraction was characterized functionally by reconstitution of fusion events in a cell-free system. This fraction (acceptor) immobilized on a solid support undergoes successive rounds of immuno-isolation. The acceptor is mixed with a post-nuclear supernatant prepared from cells with lactoperoxidase-loaded endosomes (donor) at 37°C for 45 min in the presence of ATP. Fusion is monitored by iodination of G in the lumen of acceptor vesicles with LPO delivered by the donor. The fusion is blocked at 4°C and is specific for endosomal membranes (no fusion when the acceptor is the plasma membrane fraction). The G protein can be directed to the various stages of the endosomal pathway (time, temperature, drugs). Using defined immuno-isolated fractions, we will study this pathway by combining biochemical analysis and cell-free reconstitution.

252 **BIOSYNTHESIS AND PHOSPHORYLATION OF THE POLYMERIC IMMUNOGLOBULIN RECEPTOR IN RELATION TO ITS TRANSCELLULAR ROUTING** R. Solari[1] and J.P. Kraehenbuhl[2] 1. Medical Cell Biology, Liverpool University, UK. 2. ISREC, 1066 Epalinges, Switzerland

We have investigated the processing of the polymeric immunoglobulin receptor (pIgR) in the liver, a tissue known to transport polymeric immunoglobulins (pIg) from the blood to the bile, by a combination of cell fractionation, biosynthetic labelling and Western blotting techniques and taking advantage of antibodies directed against different domains of the receptor. We demonstrate that the pIgR is synthesised as a core-glycosylated glycoprotein (Mr 105,000) on the rER, and is terminally glycosylated in the Golgi (Mr 115,000, 118,000 doublet) with a half-time of 30 mins. We show that the cytoplasmic tail of the pIgR is phosphorylated in Golgi-enriched fractions. The pIgR encounters its ligand at the sinusoidal plasma membrane and following endocytosis, the receptor-ligand complex is transported to the bile canalicular membrane. Cleavage of the pIgR membrane anchoring domain at the canalicular membrane results in the release into bile of pIg bound to the ectoplasmic domain of the receptor. The remaining membrane anchoring domain can be detected, using a monoclonal antibody directed against it, in microsomal and cytosolic fractions in addition to the bile. Consequently, the membrane anchor may be further processed and eventually eliminated in the bile.

253 TRANSEPITHELIAL TRANSPORT OF IgA IN A HUMAN INTESTINAL CELL LINE. Christian Huet, Odile Godefroy, Leslie Blair, Daniel Louvard. Département de Biologie Moléculaire, Institut Pasteur, 25 Rue du Dr. Roux, 75724 PARIS Cedex 15, France.

The mucosal immune system protects higher organisms against external pathogens such as viruses, bacteria and allergens. The underlying lymphoid tissue synthetize type A immunoglobulins. IgA dimers are transported across the epithelial cells by a specific process. To study in vitro the transport and secretion of IgA complexes we are using a unique tissue culture system. The cell line HT29 was isolated from a human colon carcinoma. In standard culture medium, these cells are undifferentiated. When galactose, instead of glucose is present in the medium, the culture displays a terminal differentiation pattern : brush borders, tight junctions and polarization of the cell monolayer are observed. We have obtained subclones of HT29 which are able to maintain their polarized properties even when cultured in glucose-containing medium. Furthermore, it is possible to grow these cultures on polycarbonate or nitrocellulose filters mounted in mini-Marbrook chambers and therefore to isolate medium covering the apical membranes from medium surrounding the basolateral side of the epithelial cells. Using such an in vitro system we are studying the receptor-mediated endocytosis of transferrin and dimeric IgA. To detect IgA receptors We developed a sensitive radioimmunoassay. Transport and secretion of IgA dimers as well as of the ectodomain of the IgA receptor (formally called secretory component) can be measured by ELISA tests. Localization of IgA and transferrin receptors are studied by immunofluorescence and immunoelectron microscopy. In undifferentiated cultures, transferrin binds to its membrane receptors, is internalized, and is later recycled back to the medium. IgA molecules are also internalized but no secretion is observed. Immunofluorescent studies show internalization of the two components in different intracellular structures. On polarized cells, IgA and transferrin receptors are localized on basolateral membranes whereas apical surfaces are negative. Intracellular pathways of transferrin and transcytosis of IgA will be discussed.

254 IMMUNOCYTOCHEMISTRY ON IGA TRANSCYTOSIS IN LIVER AND INTESTINE

H.J.Geuze and J.W.Slot
Laboratory of Cell Biology, University of Utrecht, Medical School, Nic.Beetsstraat 22, 3511 HG Utrecht, The Netherlands

Many secretions like milk, intestinal fluid, bile and saliva contain immunoglobulins (Ig), including IgA. Most of these IgA molecules occur as polymers. The pIgA is produced by plasma cells of the interstitial tissue and is translocated across the epithelial lining cells by a process called transcytosis. Transcytosis of pIgA is receptor mediated event. The IgA receptor (IgA-R), an integral membrane protein, specifically recognizes binds pIgA-ligand present us the body fluids at the basolateral sides of epithelial cells. The receptor-ligand complexes are endocytosed and transported in vesicles to the apical cell surface. Following exocytotic fusion of the IgA transfer vesicle membrane with the canalicular membrane, part of the receptor, the so-called secretory component, together with the covalently bound IgA are cleaved off from the membrane and discharged into the bile. Thus, the IgA-R is involved in only one round of ligand delivery. Many other receptors, like those for asialoglycoproteins (ASGP) in liver are re-used many times. In rat liver both IgA and ASGP ligands are taken up at the sinusoidal cell surface. However, while ASGP ligand is targetted to the lysosomes and the ASGP-R is re-routed to cell surface, IgA-R/ligand complexes are transcytosed towards the bile. Since both receptor systems are endocytosed in the same coated pits (1), pathways must separate intracellularly. Using our immunogold double-labeling method (2), we have traced the sorting organelle for ASGP-R and IgA-R in rat liver. It appeared to be the acidic prelysosomal compartment (CURL) wherein we have previously found that uncoupling and sorting of ASGP-R and ligand take place (3). CURL-tubules were found to form vesicles from local swellings which are enriched in IgA-R and bound ligand. These vesicles then travel to the bile canalicular membrane for ligand delivery.

In rat duodenum, IgA and IgA-R were observed in all cell types except endocrine cells. In human duodenum, only cylindrical cells contained IgA-R. IgA-R and ligand occurred along the lateral plasma membrane, in coated pits and vesicles and in tubules in the peripheral cytoplasm. The IgA transfer vesicles were often rod shaped and seemed to accumulate in the cell apices. Although the overall picture for IgA translocation is the same in liver and duodenal cells, differences may exist with respect to the extent that coated vesicles are involved in internalization of IgA-R-ligand.

(1) Geuze et al., 1984. Cell 37, 195-204
(2) Geuze et al., 1981. J. Cell Biol. 89, 653-655.
(3) Geuze et al., 1983. Cell 32, 277-287.

255 TRANSCYTOSIS IN THYROCYTES - THE CELLULAR BASIS FOR THE APPEARANCE OF THYROGLOBULIN IN THE BLOOD.

Volker Herzog, Department of Cell Biology, Goethestr. 33,D-8000 München 2,West-Germany

Almost 20 years ago it was shown that thyroglobulin (TG) is not confined to thyroid follicles but detectable also in the circulation. Serum concentrations of TG were found to be increased by the action of TSH. Inside-out follicles from pig thyroid gland are a useful in vitro-system to investigate the release mechanism for TG, because the apical cell surfaces of thyrocytes can be reached from the culture medium. Tracer studies with cationized ferritin have shown a transcytotic vesicle movement from the apical to the basolateral cell surfaces. Endocytosis and transcytosis are raised~10fold by stimulation with TSH and decreased by lowering the temperature. TG-gold conjugates or radiolabeled TG were used to test for corresponding transport pathways of TG. The observations show that TG is internalized by the apical cell surface and transported mainly to lysosomes; a small fraction of internalized TG is transferred across the follicle wall, bypassing the tight junctions. It is postulated that transcytosis is the cellular basis for the appearance of thyroglobulin in the blood. Gel electrophoretic analyses have shown that the molecular weight of TG is reduced from~330 kD before to~300 kD after transcytosis. It is unknown, however, whether this reduction in M_r corresponds to the compositional characteristics reported for circulating TG. The biological role of circulating TG is as yet unknown. It was observed, however, that TG is internalized and degraded by Kupffer cells and peritoneal macrophages. This points to a possible function of these cells in the formation of thyroid hormones from circulating TG. (Supported by Deutsche Forschungsgemeinschaft).

256 RECEPTOR-MEDIATED TRANSCYTOSIS OF ALBUMIN IN VASCULAR ENDOTHELIUM.

Maya Simionescu, Lucian Ghitescu, Anton Fixman, Nicolae Simionescu. Institute of Cellular Biology and Pathology, Bucharest - 79691, Romania

The uptake and transport of homologous and heterologous albumin-gold complex (Alb-Au) was investigated in several vascular beds provided with continuous endothelium. The experiments were conducted in RAP mice in which the vasculature was washed free of blood by perfusing Dulbecco's phosphate-buffered saline (PBS) at 37°C. In most experiments, bovine serum albumin-gold complex at 37°C at concentrations corresponding to A_{515}:0.1 and 0.4 (OD=0.1 cm) was perfused continuously at a flow rate of 3 ml/min for 3, 5, 10, and 30 min. In experiments on lung the tracer was given intermitently in 0.1 ml aliquots for 3, 5, 10, 15, and 35 min to a volume up to 9 ml. In all experiments after washing the unbound tracer with PBS, and fixation by perfusion, specimens were collected and further processed for electron microscopy. In additional experiments, the tracer was injected intravenously in vivo. To characterize the Alb-Au binding and its specificity, several control experiments were carried out, especially on the lung vasculature: (a) saturability experiments using Alb-Au concentrations ranging from 0.01 to 0.95; (b) competition with untagged albumin at concentrations of 0.1 to 30 mg/ml of PBS; (c) effect of high ionic strength using 0.45 NaCl; (d) competition with heparin; (e) perfusion of complexes of gold with other plasma proteins (fibrinogen, fibrinonectin), with a non-plasmic protein (glucose oxidase) or with uncharged macromolecules (polyethyleneglycol, polyvinylpyrrolydone, poly-L-proline). Most of these materials were analysed by morphometry.

The results showed that the vascular endothelia examined fell into two categories: (1) Endothelia in which Alb-Au complex is taken up and transported in relatively small amounts by fluid phase:e.g. aortic, coronary, endocardic, venous endothelium; in these locations, the tracer bound to coated pits. (2) Endothelia in which Alb-Au complex bound by absorption (1-2 rows) restrictively to uncoated pits of plasma membrane and to>95% of plasmalemmal vesicles open on the luminal front (e.g. capillary endothelium of lung, heart, diaphragm, skeletal muscle, adipose tissue). In these locations plasma membrane, coated pits and coated vesicles were not significantly marked. In these capillary endothelia, as a function of tracer concentration, 1-10% of plasmalemmal vesicles contained Alb-Au particles in fluid phase; from 5 min on, multivesicular bodies were labeled by the tracer. After 5 min of perfusion, tracer-carrying vesicles appeared on the abluminal front discharging their contents into the subendothelial space. The control experiments indicated that in the capillary endothelia mentioned, the Alb-Au binding to uncoated pits and plasmalemmal vesicles was saturable at very low concentrations and short time exposures, and was competed by albumin. No binding was obtained when Alb-Au was injected in vivo. Heparin as well as high ionic strength did not displace the bound Alb-Au complex from vesicle membrane. Gold complexes with fibrinogen, fibronectin, glucose oxidase, polyethyleneglycol,polyvinylpyrrolidone or poly-L-proline did not give a labeling pattern comparable to that of albumin.

These findings suggest that in the capillary endothelia examined, the Alb-Au complex is preferentially absorbed on specific binding sites restricted to uncoated pits and plasmalemmal vesicles. The tracer is transported in transcytotic vesicles across endothelium by receptor-mediated transcytosis, and to a lesser extent is taken up by pinocytic vesicles and delivered to multivesicular bodies. The endocytic process is not influenced by competition with unlabeled albumin. The existence of albumin receptors on these continuous capillary endothelia may provide a specific mechanism for the transport of albumin and molecules carried by this protein,such as fatty acids, steroid hormones, thyroid hormones etc. These findings also reveal that vascular endothelium is capable of sorting the permeant molecules not only according to their size and charge, but also according to their chemistry.
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257 MORPHOLOGICAL EQUIVALENTS OF MACROMOLECULAR TRANSPORT IN THE BRAIN CAPILLARIES
Ernő Dux, Ferenc Joó. Institute of Biophysics, Biological Research Center, Hungarian Academy of Sciences, H-6701 Szeged, Odesszai krt. 62. Hungary.

In our earlier investigations a specific activation of the brain capillary adenylate cyclase was found after in vitro histamine treatment and in vivo hypoxic exercises. Both histamine and hypoxia could also increase the synthesis of prostacyclin and prostaglandins in cerebral microvessels. The morphological equivalents of the increased permeability across the blood-brain barrier was followed by electron microscopy, albumin immunohistochemistry and freeze-fracture technique.

In our experiments after administering histamine via the carotid artery we found a specific increase in number of pinocytotic and coated vesicles in the capillary endothelium. The stimulation of pinocytosis was parallel with an uptake of serum albumin by endothelial cells. These effects of histamine were mediated by H_2 receptors. Beside them an accumulation of calcium in endothelial and glial cells was visualized electron cytochemically. Histamine treatment did not modify the fine structure of tight junctions as we could establish by freeze-fracture technique. On the contrary, we observed a well defined redistribution of intramembrane particles in the endothelial membranes.

Histamine injected into the cerebral ventricles could induce a reverse vesicular transport in the brain capillaries from the brain to the blood.

In the light of our results the brain edema of vasogenic type is probably realized through the activation of pinocytotic transport. This fact beside the biochemical changes underline the importance of metabolic processes in induction of macromolecular transport in the cerebral endothelium.

258 ISOLATION AND CHARACTERIZATION OF COATED VESICLES FROM FILAMENTOUS FUNGI.
T.C. Caesar-Ton That (1), K. Hoang Van (1), G. Turian (1) and H.C. Hoch (2). (1) Lab. of General Microbiology, Dept of Plant Biology, University of Geneva, Switzerland and (2) Dept of Plant Pathology, N.Y. State Agricultural Experiment Station, Cornell University, Geneva N.Y. 14456, USA.

Coated vesicles have been shown to exist in *Neurospora crassa* (Ascomycetes) and *Uromyces phaseoli* (Basidiomycetes) growing germlings. Separation of coated vesicles in both fungi was obtained when the high-speed (100.000g) pellet was fractioned on a Sephacryl S-1000 gel filtration column, according to the procedure of Mueller and Branton (J.Cell Biol.98:341-346,1984). Electron micrographs of negatively stained coated vesicles from fractions of gel filtration show the same striking lattice, coated vesicles similar to vertebrate coated vesicles. We observe two major size classes of coated vesicles in both fungi: the larger class (100-180 nm) is similar in size to vertebrate coated vesicles; the smaller class (50-80 nm) is mostly found in both fungi. When examined by SDS PAGE, the Sephacryl column fractions containing the maximum concentration of electron microscopically visible coated vesicles coincide with the bands of the protein coat of clathrin coated vesicles. The protein composition on SDS PAGE of the coated vesicles indicates a major polypeptide species of 180 Kd and minor 30-36 Kd species. Polypeptides of 100 Kd and 55 Kd are also found in the fractions containing coated vesicles.

259 RAPID INSULIN RECEPTOR CYCLING AND THE ROLE OF RECEPTOR REUTILIZATION IN CULTURED FETAL HEPATOCYTES.
Christiane Plas, Pascal Soubigou, and Eric Pringault. Laboratoire Interactions Cellulaires, Université Paris 7, UER Odontologie, 2 Place Jussieu, 75005 Paris, France

18 day-old fetal rat hepatocytes, when grown in the presence of cortisol, are characterized by : 1) a large stimulation of glycogenesis, which is maintained for 3 h ; 2) a cellular insulin degradetion which is mediated exclusively by the receptor endocytosis and inhibited by chloroquine. Comparative measurements of the rate of insulin and receptor degradation indicate that the receptor must be reutilized every 10 min (1). The insulin receptor cycle was investigated by determining the variations in cell-surface binding sites after short exposure to insulin. Cell-surface 125I-insulin receptor binding decreased sharply (by 40%) during the first 5 min of 10 nM insulin exposure at 37°C (T 1/2 = 2 min) and remained practically constant thereafter; subsequent removal of the hormone restored the initial binding within 10 min. This fall-rise sequence corresponded to variations in the number of insulin receptors at the cell surface, with no detectable change in receptor affinity. Gel electrophoresis after cross-linking of bound 125I-insulin to cell-surface proteins showed that these variations were not associated with molecular weight changes of binding components, in particular for the major labelled band Mr = 130,000. The insulin receptor cycle could be repeated several times following intermittent exposure to insulin and was both dose- and temperature-dependent. Chloroquine (70 µM) did not change the initial rate of internalization of cell-surface receptors after exposure to insulin, but decreased the proportion of internalized receptors recovered at cell-surface after removal of the hormone; it was ineffective alone. Modifications of the glycogenic response when using a unique short, or intermittent, exposure to insulin, or sequential chloroquine addition, revealed that the insulin-receptor interaction at the cell surface must be continuous, or repeated, for maximal expression of the biologial effect to occur in cultured fetal hepatocytes.
(1) Pringault E, Plas C, Desbuquois, B, and Clauser H (1985) Biol. Cell, 52: 13-22.

260 ONLY 4 % OF THE PLASMA MEMBRANE REACHES THE LYSOSOMAL COMPARTMENT PER HOUR IN CULTURED RAT FIBROBLASTS. J.-P. Draye, P.J. Courtoy, J. Quintart and P. Baudhuin (Lab. Chim. Physiol., University of Louvain and Internat. Instit. Cell. Molec. Pathol., av Hippocrate 75, B-1200 Bruxelles, Belgium).

Membrane shuttle between cell surface and lysosomes is well known, but the proportion of internalized constituents which actually transit through lysosomes may be rather small (Burgert and Thilo, Exp. Cell Res., 144, 127, 1983). To estimate the flow of plasma membrane (PM) into lysosomes, we have obtained highly purified preparations of lysosomes from cultured rat fibroblasts, and quantified (1) the association at steady-state with endogenous PM markers and (2) the kinetics of appearance of the bulk of polypeptides labelled with ^{125}I at the cell surface (Hubbard and Cohn, J. Cell Biol. 64, 438, 1975). Lysosomes were loaded with horseradish peroxidase and isolated by isopycnic centrifugation in linear Percoll gradients, followed by a 3,3'-diaminobenzidine-induced density shift (Courtoy et al., J. Cell Biol. 98, 870, 1984). Preparations with a relative specific activity (RSA) of 34 were virtually pure and contained ∿40 % of N-acetyl-β-glycosaminidase of the homogenate. Regression analysis indicates that the lysosomal compartment contributes only 1.5 % of 5'-nucleotidase but 16 % of alkaline phosphodiesterase I cell activities. When cells were radiolabelled at 4°C, the distribution of incorporated ^{125}I corresponded closely to that of 5'-nucleotidase and the peak of alkaline phosphodiesterase I. Upon reincubation at 37°C, most of the radioactivity was lost with a T1/2 of ∿20 h. By SDS-PAGE autoradiography, this kinetics was similar for all major polypeptides. A small fraction of cell radioactivity became increasingly associated with purified lysosomal preparations (yield, 52 %; RSA, 24) and leveled up at 3 % from 8 h to 25 h. By morphometry, the membrane area of the entire vacuolar apparatus and of its lysosomal compartment corresponded to 45 and 30 % of the pericellular membrane, respectively. A model which takes into account all measured parameters shows that every hour, in cultured rat fibroblasts, only 4 % of PM polypeptides reaches the lysosomes, where 88 % is degraded with a T1/2 of 0.7 h, the remainder being recycled. Although differential interiorization of PM polypeptides cannot be excluded, the model can account for the disparity in PM markers found in lysosomes on basis of different degradation rates.

261 INVESTIGATION OF Fc RECEPTOR RECYCLING ON RAT PERITONEAL MACROPHAGES

Anna L. Kiss and P. Röhlich, Laboratory I of Electron Microscopy and 2nd Department of Anatomy, Semmelweis University of Medicine, H-1450 Budapest, Hungary

During receptor-mediated endocytosis, IgG and immune complexes are internalized and digested in lysosomes. However, there are controversial data on whether Fc receptors are degraded together with the ligands or recycled to the cell surface.

In the present study rat peritoneal macrophages were incubated at 4°C with rat peroxidase-antiperoxidase /PAP/ complex for 1 h, washed and warmed up to 37°C for 5, 30 and 60 min. The cell suspensions were reincubated with new PAP at 4°C for 1 h. Cells were fixed for electron microscopy /EM/ at different stages of the experiment. In another series of experiments, the cells were preincubated with monensin /50 nM/ at 37°C for 10, 15, 20 and 30 min, then cooled to 4°C and reincubated with PAP in the presence of monensin for 1 h. After washing, the cells were processed for EM and membrane surfaces binding PAP were estimated with morphometry. In addition to EM, the bound PAP was measured spectrophotometrically.

Macrophages which had bound PAP at 4°C and were warmed up for 5 min did not show PAP on their surface membrane, all PAP was detected in endosome-like structures. Such cells, when reincubated with PAP at 4°C, again bound the ligand on the cell surface, mainly in labyrinthic invaginations. Cells which had been warmed up for longer periods /30 and 60 min/ showed the bound ligand all along the surface membrane. These findings can be explained either by Fc receptor recycling or by replenishing from a cytoplasmic pool. Such a flow of Fc receptors from the cytoplasm to the cell surface is supported by our findings that after preincubation with monensin, the ability of the plasma membrane to bind PAP was considerably reduced.

262 THE KINETICS OF LACTOFERRIN-MEDIATED IRON UPTAKE BY HUMAN MACROPHAGES AND ITS TRANSFER INTO FERRITIN. N. Moguilevsky[†], P.J. Courtoy[x] and P.L. Masson[†] ([†]Experimental Medecine Unit and [x]Laboratory of Physiological Chemistry, Louvain University, and International Institute of Cellular and Molecular Pathology, 75 avenue Hippocrate, 1200 Brussels - Belgium).

Macrophages play a central buffering role in the iron metabolism, yet how they pick up, store and release iron remains largely unknown. We have studied the lactoferrin(Lf)-mediated iron uptake and storage in human macrophages (Van Snick et al., J. Exp. Med. 146: 817, 1977) using antibodies to identify carrier proteins and to measure the kinetics of exchange. Monocytes were isolated from human blood mononuclear cells (Lymphoprep, Nyegaard) by adherence at 37°C for 1 hr in multiwell plastic dishes, and allowed to differentiate into macrophages by incubation in RPMI medium with 10 % foetal calf serum for one week. Cells were washed, and incubated for up to 120 hrs with fresh medium containing 100 µg/ml ^{59}Fe-Lf. At various intervals, cells were washed then lysed in deoxycholate and radioactivity counted. After a rapid binding (corresponding to 2.27 10^6 Fe atoms or 1.13 10^6 Lf molecules per cell), ^{59}Fe uptake increased linearly with time (0.19 10^6 atoms.cell^{-1}.hr^{-1}). Proteins carrying Fe were absorbed on latex particles coated with $F(ab')_2$ fragments of either anti-human Lf or anti-human ferritin by incubation for 3 hrs at 20°C with vigorous agitation and recovered by sedimentation. At all time points, ∿90 % of cell-associated ^{59}Fe could be recovered in the combined proteins. The Lf-associated fraction declined from 100 % at time 0, to 50 % at 18 hrs and leveled out at 10 % after 80 hrs. The ferritin-associated fraction (0 % at time 0) accounted for 50 % of cell-associated Fe at 20 hrs and 80 % at 80 hrs. Sedimentation was < 5 % when latex particles were coated with non immune IgG. Compared to the kinetics of transferrin-mediated iron uptake and delivery to cells (Octave et al., Eur J. Biochem. 115 : 611, 1981), the release of iron from Lf and its transfer into ferritin are very slow. Both kinetics correspond to that reported for the degradation of Lf by macrophages (Van Snick, et al., ibidem). This is compatible with a major pathway of Lf recycling without Fe exchange, and a minor pathway of Lf degradation in lysosomes, rapidly followed by Fe transfer to ferritin.

263 DIFFERENCES IN THE CELLULAR LOCATION OF SUBSTANCES ENDOCYTOSED BY RAT LIVER AS OBSERVED FROM THE DISTRIBUTION PATTERNS OBTAINED AFTER ISOPYCNIC CENTRIFUGATION IN A SUCROSE GRADIENT. R. Wattiaux, M.Jadot, S.Misquith, F.Dubois and S.Wattiaux-De Coninck.(Facultés Universitaires, Namur,BELGIUM).

Of the numerous substances endocytosed by the liver, some are mainly taken up by the parenchymal cells while others are taken up by sinusoidal cells. Our results indicate that centrifugation experiments can reveal information about the cellular location of a molecule endocytosed by the liver. The results show that two different distribution patterns can be observed, in a sucrose gradient for the endocytosed substance depending on whether it is taken up by parenchymal or the sinusoidal cells. The distinction between the two patterns is apparent during the different phases of its passage to the lysosomes (a) at an early time after injection; (b) during its transport to the lysosomes (c) and finally when the substance is mainly associated with these organelles. There are three main differences between the distributions.(1) The non-lysosomal structures (coated vesicles,endosomes) that contain the endocytosed molecule are denser in sinusoidal cells (median density 1.17-1.18 g/cc) than in parenchymal cells (median density 1.11-1.12 g/cc). (2) The passage from the prelysosomal to the lysosomal compartment gives rise to a bimodal distribution of the compound if it is present in parenchymal cells, on the other hand, during the same period, if the substances are taken up by the sinusoidal cells, the distribution curve progressively migrates towards higher density regions without being bimodal. (3) When the molecule is endocytosed by parenchymal cells, its lysosomal distribution is not affected by a previous injection of invertase (900µg/100 g body wt of rat); however the lysosomal distribution is shifted towards higher density regions if the compound is situated in the sinusoïdal cells. Within each group of substances tested by us, there exists obviously, some individual differences between the distributions of the compound, which are probably due to factors such as the different rates of penetration into the cells and the intracellular degradation of the molecules. Nevertheless, these differences are not sufficient to significantly changes the characteristics of the distribution patterns.

264 THE GAL-SPECIFIC RECEPTOR SYSTEM IN RAT LIVER CELLS DURING DEVELOPMENT

Laura Conti Devirigiliis[1], Salvatore Russo-Caia[2] and Luciana Dini[2]

(1)Department of Cellular and Developmental Biology, University of Rome "La Sapienza".P.zale A.Moro 00185 Roma - ITALY

(2)Department of Biology - 2nd University of Rome, Via O.Raimondo - 00173 Roma (Italy)

The number and distribution of gal-specific binding sites was investigated in rat liver during prenatal development. In "in vitro" and "in situ" experiments ligand binding to hepatocytes, liver macrophages and endothelial cells was followed by electron microscopy using Lac-BSA adsorbed onto 5 nm colloidal gold as ligand.

The binding capability, starting in a late stage of fetal development, is very low for both hepatocytes as well as macrophages, that show a statistically distributed arrangment of sites. On the contrary, bounded particles are absent on endothelial cell surface, which is also lacking of the typically coated regions.

Experiments carried out "in vivo" at 37°C indicate that the endocytosis occurs for some extent.

These results, together with the data previously obtained for the post-natal development, suggest that the expression of galactose specific receptor activities on the different liver cells follows a differentiation pattern independently regulated.

265 ENDOCYTOSIS IN HEPATOCYTES AND LIVER ENDOTHELIAL CELLS.

Grete M. Kindberg, Winnie Eskild, Trond Berg. Institute for Nutrition Research, University of Oslo, Postbox 1046, Blindern, 0316 Oslo 3, Norway.

The intracellular transport and degradation of endocytosed protein ligands taken up by receptor-mediated endocytosis in isolated rat liver parenchymal and endothelial cells were studied by subcellular fractionation. Fractionation was done in Nycodenz and sucrose gradients. The ligands chosen were asialoorosomucoid (in parenchymal cells) and formaldehyde-treated albumin (FSA) (in endothelial cells). By using ligands labeled with ^{125}I-tyramin-cellobiose the labeled degradation products were trapped in the lysosomes, and their density distributions could therefore be used to identify these organelles in subcellular fractionation experiments.

The results showed that the metabolism of the ligands could be divided into prelysosomal and lysosomal steps for both parenchymal and nonparenchymal cells. Both asialoorosomucoid and FSA were initially (< 1min) in slowly sedimenting, small vesicles and then in endosomes with higher bouyant density and/or size. In parenchymal cells degradation was initiated in the endosomal compartment and continued in denser lysosomes. Uptake of ligand in the lysosomes in endothelial cells was very rapid; therefore degradation of labeled ligand could be studied in isolated lysosomes in vitro.

The rate limiting steps for intracellular degradation were different for the two cell types. In the steady state most of the undegraded ligands were in endosomes in the parenchymal cells and in lysosomes in the endothelial cells.

266 INTRACELLULAR PROCESSING OF RICIN BY ISOLATED ZAJDELA HEPATOMA CELLS AND NORMAL RAT HEPATOCYTES. M. Decastel. Unité 180 INSERM, CNRS UAC 81, UER biomédicale des Saints-Pères, 45 rue des Saints-Pères, 75006 Paris.

Membrane surface components are particularly involved in morphogenesis, growth control and cell differentiation.
Membrane dynamics can be analyzed by studying the interaction between cells and effector molecules. Thus, we investigate the binding, uptake and degradation of the toxin isolated from Ricinus communis, RCA II, to Zajdela hepatoma cells and normal rat hepatocytes.
Hepatoma cells are 30-fold more sensitive to the toxic effects of ricin than normal cells. Binding data show that hepatoma cells have a greater number of toxin-binding sites than normal cells, while the apparent association constant of the toxin to the surface receptors appear similar. Furthermore, preliminary kinetics studies indicate that internalization of ricin in hepatocytes seems to be slower than in hepatoma cells. This suggest that others factors are involved in the sensitivity of hepatoma cells to ricin.

267 PRESENCE AND TRANSIT OF LIPOPROTEIN RECEPTOR THROUGH ENDOCYTIC VESICLES IN ONE GERMINAL AND ONE SOMATIC TYPE OF CELLS.

C.de PAILLERETS, A.ALFSEN, M.BOMSEL and H.WEINTRAUB
CNRS n°64, U.E.R. Biomédicale des Saints Pères - 45 rue des Saints Pères 75006 PARIS France

In adrenocortical cells, the presence of a LDL receptor has been demonstrated in plasma membrane and in two different populations of morphologically different coated vesicles. The preparation and isolation of each type of vesicles has allowed the comparison of their protein and lipid composition. The cellular organelles from which they derive have been defined by the analysis of specific enzymatic transformed substrates present in each type of vesicles.
In oocytes the presence of the vitellogenin receptor has been demonstrated in some of the cellular compartments obtained by density gradient fractionation. In the vitellogenic stages of oogenesis these compartments have been characterized as endocytic vesicles and transient yolk bodies which are the precursor of the yolk platelets.
The technic used for the demonstration of the presence of the receptor was ligand blotting and ELISA detection in both types of cells studied.

268 SOME FINE STRUCTURAL CHARACTERISTICS OF CHOLESTEROL TRANSPORT IN HUMAN AND RAT ADRENOCORTICAL CELLS IN VITRO. Ida E. Tóth, D. Szabó, Katalin Sz. Szalay, Angéla Gyévai. Institute of Experimental Medicine Hungarian Academy of Sciences. H-1450 Budapest, POB 67, Hungary

Steroid-secreting cells are unique in respect of their cholesterol demand, because they use cholesterol for hormone production and store cholesterol in lipid droplets. The main sources of plasma cholesterol: the low-density lipoproteins (LDL) and the high-density lipoproteins (HDL) were labelled with colloidal gold of different diameter in order to visualize cholesterol transport. Human adrenocortical cells of surgically removed hormone producing adenomas were incubated with labelled LDL and HDL (LDL-Au and HDL-Au) for 1 and 24 h at 37 ^{0}C, then processed for electron microscopy. LDL-Au were accumulated in coated pits, coated vesicles, non-coated vesicles and lysosomes via receptor mediated endocytosis. HDL-Au were rarely observed on the surface of the cells or within subcellular organelles, even after a long time incubation. Qualitative differences were not observed between the normal adrenocortical cells and the adenomatous cells in respect of their cholesterol transport. Adrenocortical cells of rats were treated with LDL-Au and HDL-Au for 1, 6 and 24 h at 37 ^{0}C then processed for electron microscopy. LDL-Au were accumulated by the cells only after a 24-h incubation. HDL-Au were bound and internalized both by zona glomerulosa and zona fasciculata cells even after 1 h incubation: they were present on the surface of the parenchymal cells, often attached to the membrane of microvilli, but rarely in coated pits and coated vesicles. HDL-Au were observed in non-coated vesicles, multivesicular bodies and lysosomes. Steroid hormone assays were performed to reveal the steroidogenic effect of lipoproteins.

269 COMMON ENDOCYTIC PATHWAY OF EPIDERMAL GROWTH FACTOR AND TRANSFERRIN TO ACIDIC (pH 6.1) PARA-GOLGI COMPARTMENT IN A431 CELLS. A.D.Sorkin, L.V.Teslenko, N.N.Nikolsky. Institute of Cytology, Academy of Sciences of the USSR, 194064, Leningrad, Tichoretsky pr.,4, USSR

To study a mechanism of intracellular sorting of ligand-receptor complexes the endocytosis of epidermal growth factor (EGF) and transferrin was investigated on A431 cells by using microscope spectrofluorimetry and video intensified microscopy. Rhodamine-labeled EGF (EGF-Rh) and fluoresceine-labeled transferrin (TF) were simultaneously added into culture medium at 37°C. After 15-20 min both ligands accumulated in great (3-6 μm) juxtanuclear structure consisted of close-related vesicles. This bright compartment was shown to be associated with Golgi complex. The co-localization of EGF-Rh and TF in endosomes and para-Golgi was demonstrated with the help of double-label fluorescence technics. The measurements of pH of TF microenviroment on single cells showed that the pH value of 6.1± 0.3 is actively maintained in para-Golgi vesicles. Using fluoresceine dextran we obtained a lysosomal pH of 5.0±0.2 in A431 cells. Our results indicate that EGF as well as transferrin presumably remain associated with receptor during endocytosis in A431 cells up to being transfered into medium with pH of 5.0, that is to lysosomes. It was concluded that para-Golgi is just compartment responsible for intracellular sorting of internalized ligands and receptors

270 PROTEOLYTIC PROCESSING OF LH/hCG RECEPTOR-HORMONE COMPLEX IN CULTURED RAT LUTEAL CELLS. Sakari Kellokumpu and Hannu Rajaniemi, Department of Anatomy, University of Oulu , SF-90220 Oulu , Finland.

The catabolic fate of the LH/hCG receptor-hormone complex in cultured rat luteal cells was studied. The cells were pulse-labelled at 4°C with ^{125}I-hCG, and subsequently incubated at 37°C. At specified times, the cells were treated with acidic buffer (pH 3) to quantitate the surface-bound and internalized hormone. The radioactivity released into the medium was also counted and subjected to TCA-precipitation to monitor the extent of hormone degradation during the 12-h incubation period. The cells were found to internalize the hormone at a very slow rate as at all times examined only 10% of the radioactivity remained associated with the cells after low pH treatment. The appearance of the hormone degradation products in the medium was comparable to the low internalization rate of the hormone. However, most (65-75%) of the radioactivity released into the medium was TCA-precipitable. Chemical cross-linking and analyses by SDS-PAGE of this material revealed that the cells released, in addition to intact hCG and its degradation products, also two previously characterized receptor fragment- ^{125}I-hCG complexes (M$_r$ 96 000 and 74 000, Kellokumpu and Rajaniemi, 1985, Endocrinology 116;707-714). These complexes could not be detected inside the cells, suggesting that they were released directly from the cell surface. The results indicate that in contrast to several other receptor-ligand systems or cells studied, the degradation of the LH/hCG receptor-hormone complex in rat luteal cells does not necessarily require their internalization but it may also occur already at the cell surface. This mechanism thus provides an additional possibility by which receptor down-regulation may occur.

271 LH AND HCG : A COMMON RECEPTOR, A DIFFERENT FATE. Nicole GENTY (1), Roland SALESSE (1), Françoise DACHEUX (2), Jean GARNIER (1). (1) Laboratoire de Biochimie Physique, Université Paris-Sud, 91405 ORSAY (FRANCE), (2) Station de physiologie de la Reproduction, INRA, 37380 MONNAIE (FRANCE)

LH (the physiological hormone) and hCG (the human chorionic analog) share the same hormonal receptor at the surface of testicular Leydig cells. Both hormones elicit the same steroidogenic response in these cells. Though hCG is widely used as a substitute for LH we found important differences in the fate of the receptor hormone complex. We compared by radiohormone receptor titration the number of receptors present on the cell surface under stimulation by the two hormones at 35°C.

The physiological hormone, pLH, produced a rapid dose dependant decrease in the total number of the gonadotropin receptors present on the cell surface. This decrease was reversible upon hormone removal, receptors were recycled in a free state on the cell surface. The round trip of the receptor in LH stimulated cells took 15 to 20 minutes. For period of incubation up to 2 hr, hCG up to 5 μg/ml did not induce any internalisation of the gonadotropin receptors. The hCG receptor complexes remained at the cell surface even during a chase.

These data concerning the gonadotropin receptor are in agreement with previous results showing that LH, but not hCG was internalized and degraded by Leydig cells in primary culture. These data are compatible with preliminary ultrastructural observations showing that gold LH enters the cells through coated pits, and is internalized in coated vesicles and large vacuoles probably endosomes, while gold hCG is maintained on the cell surface.

272 TESTOSTERONE MEDIATED ENDOCYTOSIS IN HAMSTER SEMINAL VESICLE SECRETORY CELLS.
Lucinda R. Mata and J.F.David-Ferreira. Department of Cell Biology, Gulbenkian Institute of Science, Oeiras

The secretory cells of hamster seminal vesicle display endocytosis and, after castration, their secretory activity is decreased and exocytosis is relatively more reduced than secretory protein production (1,2,3). This work is aimed to investigate the effect of decreased exocytosis on endocytosis in hamster seminal vesicle secretory cells after testosterone withdrawal.

The seminal vesicles from 8-day castrate animals and those from controls were emptied, set ut in vitro and 6% HRP in saline was injected into their lumens. The tissue was processed for EM after 5, 20, 40 and 60 minutes incubation (1). One gland from each of three castrates and one gland from each of three control animals were studied at each time interval.

The observations show that HRP is taken up by endocytic vesicles and transferred to multivesicular bodies, secretory vacuoles, Golgi cisternae, intercellular and sub-epithelial spaces, both in castrate and control secretory cells. Nevertheless, the labeling of each one of those compartments is lighter in castrate secretory cells than in control ones. The labeling of Golgi cisternae, coinciding with the highest number of labeled endocytic vesicles, is observed after 40 and 20 minutes incubation respectively in castrate and control cells. Although the endocytic pathway is the same in castrate and control cells, the kinetics of labeling is delayed in castrate secretory cells when compared to that of control ones.

These results are consistent with endocytosis decrease in castrate seminal vesicle secretory cells and suggest endocytosis coupled exocytosis mediated by testosterone.

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273 LOCALIZATION AND INTERNALIZATION OF COLLOIDAL-GOLD LABELLED INSULIN IN HORMONALLY IMPRINTED CELLS.
B. Madarász, Edit Bohdaneczky and G. Csaba. Department of Biology, Semmelweis University of Medicine, Budapest, Hungary

The influence of hormonal imprinting /the first, decisive encounter of the cell with the hormone/ on the internalization and intracellular localisation of insulin has been studied in Chang liver cell cultures, and colloidal-gold labelled hormone was used for detection. There was a moderate binding of hormone in coated pits of untreated cells and in vesicles covered by smooth membrane containing the insulin-gold complex in the cytoplasm. At the same time, many coated pits and coated vesicles were characteristic for the cells pretreated /imprinted/ by insulin. Later the insulin-gold appeared in lysosomes of untreated cells, while in lysosomes, freely in the cytoplasm, on the nuclear membrane and in the nucleus of the imprinted cells. The experiments provide data to the internalization of insulin-receptor complex and to the mechanism of hormonal imprinting alike.

274 BINDING, INTERNALIZATION AND OVERLAP OF COLLOIDAL GOLD LABELLED FSH AND TSH IN CELL CULTURES OF GONADS
Magdalena Gruszczynska, Ottilia Török, F. Sudár and G. Csaba
Department of Biology, Semmelweis University of Medicine, Budapest, Hungary

In earlier experiments an overlap between gonadotropins (FSH-LH) and thyrotropin (TSH) has been observed by using FITC-labelled hormones or functional indexes. In the present study testis and ovary cells of newborn CFY rats and Chinese hamster ovary (CHO) cell line has been studied, using colloidal gold labelled hormones. FSH and TSH bound alike to the membrane of the cells demonstrate the overlapping binding of these two different hormones in the critical perinatal period of receptor development. The hormones have been localized in coated pits and vesicles and have been transported in smooth membrane coated vesicles. After 30 or 60 minutes of treatment, lysosomal localization has also been observed parallel to the membrane binding.

275 INTERNALIZATION OF TRIIODOTHYRONINE (ELECTRONMICROSCOPIC STUDY)
Zdeněk Kostrouch(1), Ivan Raška(2), Vladimír Felt(3), Ema Holečková(1), Jara Nedvídková(4), Marta Kostrouchová(5). (1) 1st Inst.Pathol.,Charles Univ., Prague, (2) Inst.Exp.Med.,Czechoslovak Acad.Sci., Prague, (3) Inst.Hlth., Prague 4, (4) Res. Inst.Endocrinol., Prague, (5) Faculty Pediatr.,Charles Univ., Prague.

Mechanisms of triiodothyronine (T_3) entry and movement in the cells are not precisely known. It has been recently suggested, that it might be receptor-mediated endocytosis. We have investigated T_3 internalization in human peripheral leukocytes using ultrastructural autoradiography and ultrastructural affinity cytochemistry. The cells were incubated at 37°C for 5 and 60 minutes with (^{125}I)T_3 or with colloidal gold stabilized with conjugate of T_3 and bovine serum albumin (GT_3A). In both cases competitive controls with free unlabeled T_3 were performed. Morphometric analysis of sets of autoradiograms shows specific binding of T_3 in plasma membrane for 5 and 60 minutes of incubation. Affinity cytochemistry proved specific binding of GT_3A on plasma membrane. Solitary particles or clusters were internalized into plasma membrane invaginations. Particles were frequently found in the Golgi, lysosomes and, less often, in endoplasmic reticulum. Morphometric analysis proved specific GT_3A internalization for 5 minutes of incubation time. For 60 minutes incubation, the specificity of internalization could not be demonstrated by competition. The results indicate that T_3 binds specifically to the plasma membrane and is internalized via receptor-mediated endocytosis or another kind of adsorptive endocytosis. However, T_3 is probably partially internalized via another non-specific mechanism stimulated by the presence of free T_3.

276 A QUANTITATIVE FLOW CYTOFLUOROMETRIC ANALYSIS OF ENDOCYTOSIS.
Patrick MIDOUX, Annie-Claude ROCHE and Michel MONSIGNY
Biochimie Cellulaire et Moléculaire des Glycoconjugués, C.B.M., CNRS, 1, rue Haute, F-45071 Orléans cedex 2.

Membrane lectins (1) which occur in many normal cells and tumor cells have been evidenced at the surface of Lewis lung carcinoma cells (3LL cells) (2) by using fluorescein-labelled neoglycoproteins (3). The flow cytofluorometer and the specific fluorescence intensity of F-neoglycoprotein have been standardized (3). Standardization of the FACS Analyzer was achieved by using 1-(fluoresceinyl thioureido)-4,8-diaza-eicosane bound to anionic polystyrene beads. The quantum yield of fluorescein bound to a protein is dependent on the number of fluorophore molecules bound to a protein molecule and on the pH of the environmental medium. The mean fluorescence intensity of a fluorescein molecule bound to a protein decreases when the number of fluorescein residues per molecule increases ; however, after proteolytic digestion the mean fluorescence intensity of a fluorescein molecule is that of free fluorescein. When the cells are incubated in the presence of F-neoglycoprotein, at 37°C, the fluorescence intensity of a cell which is rather low because of the low pH of endosomes and lysosomes can be increased several times upon a post incubation at 4°C in the presence of monensin, a proton/sodium ionophore. The intensity of the fluorescence associated with a cell after monensin post-treatment is shown to be dependent on the digestion process occurring during the endocytosis at 37°C. At 4°C, 3LL cells bind 750 000 molecules of fluorescein-neoglycoprotein with an apparent binding constant of 2 x 10^6 l.mole^{-1}. At 37°C, after 4 h of incubation 2.2 x 10^6 molecules of neoglycoprotein were cell-associated, among them at least one third were degraded. (1) Monsigny, M. et al., Biol. Cell, 47, 95-110 (1983). (2) Roche, A.C. et al., J. Cell. Biochem., 22, 131-140 (1983). (3) Monsigny, M. et al., Biol. Cell., 51, 187-196 (1984).

277 EFFECT OF TWO CYTOSKELETON INHIBITING DRUGS ON THE ENDOCYTOSIS OF SUCROSE BY THE RAT LIVER IN VIVO.
M.-F. Ronveaux-Dupal, R. Thibaut-Vercruyssen, Y. Poumay and M. Pypaert. Unité de Cytologie, Facultés Universitaires Notre-Dame de la Paix, 61, rue de Bruxelles, 5000 Namur. Belgium.

In vivo, sucrose is taken up into rat liver cells by fluid-phase pinocytosis and is led to the lysosomes where it accumulates causing a progressive vacuolization of the cells (Wattiaux R. 1966. Thesis, Université de Louvain). By the use of colchicine and cytochalasin B (CB), we wanted to investigate the role of the cytoskeleton in this process. We realized an ultrastructural study with acid phosphatase cytochemistry, and a biochemical study measuring the activities of two lysosomal enzymes (acid phosphatase and β-galactosidase) as well as sucrose concentration in the liver homogenate and the high-speed supernatant of a part of the homogenate (39000 RPM for 40 min.). 2 or 3 hours after an intraperitoneal injection of sucrose (8 ml 50% w/v), the lysosomes became swollen and we observed a net increase in the unsedimentable activities of the two lysosomal hydrolases as well as large amounts of sucrose in the two fractions. When colchicine (0.1 mg/100 g body weight) was injected before sucrose injection, we observed an inhibition of the sucrose uptake in the liver greater than with CB (0.038-0.15 mg/100 g body weight). In the colchicine-treated rat hepatocytes, a great number of non lysosomal small vesicles, probably sucrose pinosomes, were lying near the sinusoidal membrane while the lysosomes were disseminated throughout the whole cytoplasm. It seemed that, after the antimicrotubular drug treatment, the cytoplasmic vesicles move randomly, delaying fusions with other vesicles. So the sucrose uptake's inhibition could be explained by a delay in the encounter of pinosomes with lysosomes and/or in the plasma membrane recycling. With CB, we observed, within the lysosomes, the appearance of light inclusions which we believed to be lipid inclusions resulting from fusions of VLDL-containing secretory vesicles with lysosomes. This suggests that the partial inhibition of sucrose endocytosis was not caused by a direct effect of the drug on the sucrose uptake but rather by its effect on the recycling of plasma membrane.

278 HORMONAL REGULATION OF THE UPTAKE OF YOLK PROTEINS IN THE OVARIES OF AN INSECT, *DYSDERCUS CINGULATUS* /HEMIPTERA/.

Adrien Fónagy /1/, László G. Kőmüves /2/, Zsuzsanna Móricz /2/, Miklós Sass /2/. /1/ Research Institute for Plant Protection, Hungarian Academy of Sciences, H-1525 Budapest, POB 102; and /2/ Department of General Zoology, ELTE University, H-1088 Budapest, Puskin u. 3.; Hungary.

The oocytes of insects accumulate a large amount of yolk proteins from the haemolymph during vitellogenesis. We have studied the hormonal regulation of uptake of yolk proteins by the oocytes in the red cotton bug, *Dysdercus cingulatus* during development and following treatments with *Precocene II* /PII/ and an analogue of *juvenile hormone* /JHA/.

Formation of coated pits on the plasma membrane and the accumulation of endocytotic vesicles and yolk granules in the cytoplasm of oocytes were observed during vitellogenesis. Continuous contact treatment of imagos with PII prevented these processes. Gel electrophoretic analysis of the polypeptide pattern revealed accumulation of vitellogenic polypeptides in the developing eggs and their complete absence in the oocytes of PII treated females. JHA treatment, given in vivo to PII treated bugs or in vitro to ovaries of PII pretreated females led to the appearance of endocytotic vesicles in the oocytes.

Our results seem to support the view that endocytotic uptake and accumulation of yolk proteins by the oocytes is regulated by juvenile hormone in *Dysdercus cingulatus*.

279 UPTAKE OF STORAGE PROTEINS BY THE INSECT FAT BODY AND ITS HORMONAL REGULATION

M.Sass, L.Kömüves, E.Fellinger, G.Réz, J.Kovács. Dept. of Gen. Zoology, ELTE Univ., H-1088 Budapest, Puskin u.3., Hungary

Storage protein was isolated from the haemolymph of Mamestra brassicae by gel permeation chromatography on Sepharose 6B column. On SDS-PAGE this protein was shown to consist of subunits giving two bands with apparent m.w.s. of 87 000 and 92 000. The concentration of storage protein decreases sharply at the beginning of the prepupal stage. At the same time multivesicular bodies appear in the fat body cells which transform later into protein storage granules. When the fat body was fractionated by ultracentrifugation the main protein in the fraction of protein storage granules was identical with the storage protein isolated from the haemolymph. The exogenous 20-hydroxyecdysone treatment simultaneously stimulates the formation of protein storage granules in the fat body and the decrease of haemolymph protein concentration. It seems therefore that the moulting hormone plays an essential role in the selective endocytosis of the storage protein by the prepupal fat body.

It is very notable that a well detectable amount of 20-hydroxyecdysone was found in the protein granule fraction, however its role and fate in metamorphotic processes needs further investigations.

280 ENDOCYTOTIC MECHANISM OF NA-HUMATE AND CHINESE TANNIN UPTAKE BY ROOT AND COLEOPTILE CELLS OF WHEAT SEEDLINGS.

Andrzej Tretyn, Copernicus Univ.,Inst.Biology, 87-100 Toruń, Poland

Water solution of Na-humate isolated from soil caused a 50% increase in length of roots in wheat seedlings. It effected changes in the cellular ultrastructure of the cortex cells of root apexes. Those changes were reflected through the formation of invaginations of the plasma membrane and of small cytoplasmic vacuoles, and through the appearence within the central vacuoles of double-membraned so-colled secondary vacuoles. A similar process was observed in cells of wheat coleoptile section soaked in Na-humate and Chinese tannin solutions. After being stained with $FeCl_3$ the structures in question were found to contain tannin molecules.

The results of the experiment allow the conclusion that the formation of invaginations in the plasma membrane, of cytoplasmic vacuoles and secondary vacuoles effected by Na-humate and Chinese tannin is due to the heterophagic activity of plant cells. These structures constitue consecutive stages of endocytotic uptake of these macromolecular organic compounds and their storage inside cellular vacuoles.

281 EFFECT OF CHOLESTEROL-PHOSPHOLIPID LIPOSOMES ON FOOD-VACUOLE FORMATION IN TETRAHYMENA PYRIFORMIS. Hanna Fabczak. Institute of Experimental Biology, Department of Cell Biology, 02-093 Warszawa, 3 Pasteur Street, Poland

The food-vacuole formation rate and kinetics of cholesterol incorporation into cells were investigated in Tetrahymena pyriformis during incubation in a suspension of cholesterol containing phospholipid liposomes and carmine, a phagocytosis-inducing agent. Treatment of the ciliates with liposomes at cholesterol to lecithin ratio 0.7 caused a gradual increase of phagocytic activity while at ratio of cholesterol to lecithin of 1, transient enhancement of the activity was followed by strong long-lasting inhibition one. The phagocytic activity inhibition was completely irreversible, despite of liposomes removal from cell medium. For cholesterol determination effectively incorporated into Tetrahymena cells, the amount of cell-associated radioactive cholesterol derived from cells was measured. Obtained data showed rapid uptake of cholesterol molecules occuring within first two hours, after which uptake levels off, reaching a plateau. At the plateau level amount of cell incorporated cholesterol attains the value of 0.06 uM per mg of cell protein. The observed correlation between cholesterol uptake and phagocytosis inhibition in Tetrahymena cells suggests that cell-associate cholesterol seems to modify the functional membrane properties in the cell.

282 ADSORPTIVE AND CONCENTRATIVE ENDOCYTOSIS OF THE LOW-MOLECULAR, FLUORESCENT DNA LIGAND - DAPI BY PARAMECIUM OCTAURELIA CELLS.
Bogna Skoczylas, M. Nencki Institute of Experimental Biology, Polish Academy of Science, 02-093 Warsaw, 3 Pasteura Str., Poland

Paramecium cells incubated in DAPI solution, at 27°C, showed a bright fluorescence of the nuclei indicating the presence of the drug therein. At the temperature of 6°C, while phagocytosis was completely inhibited, no fluorescence in nuclei and no food vacuoles in cytoplasm of Paramecium could be observed. However, when these cells were carefully washed and then the incubation was continued at 27°C in a new DAPI-free medium the fluorescence in nuclei appeared. These results suggest that at a low temperature DAPI molecules are firmly adsorbed on the plasma membrane. But, at the thermal conditions favourable for a phagocytotic uptake of the drug, the quantity of molecules adsorbed on the cell surface appeares to be sufficient to evoke fluorescence in the nuclei.

Furthermore, it was stated that the concentration of DAPI in the food vacuole compartment, calculated after 15 min of the drug uptake, was thousand times higher than DAPI concentration in the cells surrounding medium. The presented results suggested that the uptake of these strongly positive, bivalent molecules occurs by an adsorptive and concentrative endocytosis.

283 ENDOCYTOSIS IN PARAMECIUM CELLS MODIFIED WITH POLYANIONS AND POLYCATIONS
Elzbieta Wyroba, Department of Cell Biology, Nencki Institute of Experimental Biology, 02-093 Warsaw, Poland

Polyamine - spermine, poly-L-lysine and poly-L-glutamic acid have been used as the agents modifying the phagocytic activity of Paramecium cells. The physiological investigations have been correlated with ultrastructural observations on the cell membrane and cytopharyngeal region. Spermine at 1-1.5 uM concentration has been found to inhibit completely the phagocytic activity in the starved cells and diminish it significantly even when applied at very low dose (0.005 uM) whereas the cells modified with the poly-L-lysine and poly-L-glutamic acid demostrated a slight change in the uptake of latex particles as compared with control. Binding of surface marker ruthenium red on the cell membrane was not homogenous in the latter cases: "droplets" of the reaction product ($RR\text{-}OsO_4$) may be observed, contrary to the untreated samples. In the spermine-pretreated cells bundles of filaments have been formed beneath the cell surface and cytopharyngeal membrane analogous to those observed by the others on the isolated samples of actin incubated with spermine. Such bundles have not been found either in the control nor in the poly-L-lysine and poly-L-glutamic treated cells. It seems therefore that spermine-induced inhibition of phagocytic activity may be correlated with the observed phenomenon whereas the interaction of poly-L-lysine and poly-L-glutamic acid with Paramecium cell membrane - under the experimental conditions tested - is less efficient in the modifying of endocytosis.

284 ADRENERGIC AND HISTAMINERGIC STIMULI INCREASE THE CAPACITY FOR CATION-INDUCED PINOCYTOSIS IN AMOEBA PROTEUS. Jan-Owe Josefsson, Department of Pharmacology, University of Lund, Sölvegatan 10, S-223 62 Lund, Sweden.

Cations induce pinocytosis by associating with negative groups in the glycocalyx and the plasma membrane of the amoeba. This reaction removes Ca^{2+} from surface sites, depolarises the membrane, causes the entry of Ca^{2+} into the cell and initiates the formation of pinocytotic channels.

The pinocytotic response to Na^{+} is reduced in starved amoebae but their capacity for pinocytosis can be activated by various chemical stimuli (Exp. Cell Res. 154. 376. 1984, Peptides 6. suppl. 3. in press).

The activating effect of adrenergic and histaminergic agents was studied in Pringsheim-cultured, Tetrahymena-fed Amoeba proteus starved for 10 days. The drugs were dissolved in the inducing solution (100 mM NaCl, pH 6.9) and pinocytosis was determined by counting the pinocytotic channels using phase-contrast microscopy. Several adrenergic agents activated the amoeba. The following rank order of potency was found: (±) procaterol (10^{-10} M) > (±) pindolol (10^{-9} M) > (-) isoproterenol (10^{-8} M) > (-) adrenaline > (-) noradrenaline > (±) terbutaline (10^{-7} M). It resembles the order for stimulation of the β_2-receptor in mammalian tissues. The effect of the drugs was diminished by the β_2-selective antagonist ICI 118551. (-) Pindolol and (-) terbutaline were 1000 times more potent than their (+) isomers suggesting action on adrenergic membrane receptors in the amoeba akin to the β_2-subtype in mammalian cells. Histamine analogs were less effective. Most potent was the H_2-agonist impromidine (10^{-8} M) while histamine was effective only in the presence of the H_1-antagonist mepyramine. These activating effects were counteracted by the H_2-blocker metiamide, suggesting that agents which act on different subtypes of histamine receptors mediate opposite effects on pinocytosis. Although the mechanism by which biogenic amines enhance the capacity for pinocytosis is unknown the data suggest that the amines interact with specific receptors in the amoeba membrane.

285 UPTAKE OF HORSERADISH PEROXIDASE BY AMOEBA PROTEUS. Jan-Owe Josefsson (1), Maria Nilsson (1), Gösta Arvidson (2). (1) Department of Pharmacology, University of Lund, Sölvegatan 10, S-223 62 Lund, Sweden and (2) Biomedical Center, Department of Physiological Chemistry, Box 575, S-751 23 Uppsala, Sweden.

Horseradisn peroxidase (HRP), determined by its enzymatic activity, was taken up by Amoeba proteus. Low concentrations of the enzyme (< 10 ug/ml) was interiorized by adsorbtive pinocytosis whereas high concentrations indicated fluid pinocytosis. The molecular charge of HRP influenced binding of the enzyme to the amoeba membrane. Thus, cations displaced HRP from the cell-surface and reduced the uptake while inhibitors of mannose receptors had no effects. Uptake of an acidic isoenzyme of HRP which bound to mannose-sensitive sites in the membrane was slow and little influenced by cations in the medium. The kinetics of uptake of HRP was not linear with incubation time. Deviation from linearity may indicate constant exocytosis of HRP. Uptake, but not binding of HRP, was increased by phorbol myristate acetate (10^{-9}M).

Chilling the cells to 7°C increased the rate of uptake of HRP four times. Uptake followed bulk phase kinetics. It required well nourished cells in a calcium containing medium (100 uM) and was further increased at low pH (pH 4.3). When normal temperature was restored HRP was released into the medium and binding of the enzyme to the membrane increased. Conditions optimal for intracellular accumulation of HRP were identical to those which cause the cells to secrete a lysolecithin-like factor (PRF) which regulates cation-induced pinocytosis in the amoeba (Eur. J. Cell Biol. 28. 27 and 34. 1982). Both the release of PRF from the amoeba and its uptake of HRP in the cold were reduced by two inhibitors of phospholipase, mepacrine (50 uM) and p-bromophenacylbromide (1 uM). Cation-induced pinocytosis is blocked by these drugs (Acta physiol. scand. suppl. 508. 37. 1982) and so the present data suggest that activation of phospholipase may be the mechanism by which the amoeba regulates pinocytosis.

286 ENDOCYTOSIS IN DICTYOSTELIUM DISCOIDEUM AMOEBAE

Gérard Klein and Michel Satre, Laboratoire de Biochimie, Centre d'Etudes Nucléaires, 85X, 38041 Grenoble cedex, France.

Fluid-phase pinocytosis is a very active process in the amoebae of the slime mold Dictyostelium discoideum. Kinetics of pinocytosis in growth medium were investigated over an extended period of time (up to 6 hours) in D. discoideum using the fluid-phase marker fluorescein isothiocyanate (FITC)-dextran. FITC-dextran added to the medium accumulated rapidly inside the cells with a rate of influx equivalent to 9 μm^3 of fluid/cell x min. After a period of about 90 min of linear uptake, the intracellular FITC-dextran reached an equilibrium with that present in the external medium. This dynamic equilibrium could be maintained for several hours and corresponded to a strict balance between the pinocytosis and exocytosis as shown by efflux measurements and pulse experiments with (^{3}H)dextran. FITC-dextran was concentrated intracellularly as ascertained by the fact that at equilibrium the amount of internalized FITC-dextran reached a value equivalent to 600-800 μm^3 of fluid taken up per cell, i.e. a volume higher than the total aqueous space of the cell (520 μm^3).

The compartment in which the intracellular FITC-dextran was stored could be completely washed out when free FITC-dextran was removed from the external medium.

Vanadate, an inhibitor of plasma membrane ATPase, inhibited growth of D. discoideum amoebae at a concentration of 1 mM and blocked totally pinocytosis of FITC-dextran. This occurred by a selective reduction of fluid influx as, in cells equilibrated and maintained in the presence of external FITC-dextran, vanadate induced complete reexport of the internal fluid-phase marker.

287 DISTRIBUTION OF Ca-ATPase ACTIVITY AND OF CALCIUM BINDING SITES ON PLASMA MEMBRANE DURING PHAGOCYTOSIS IN AMOEBA

Stanisława K.Głowacka, Andrzej Sobota. Nencki Institute of Experimental Biology, Polish Academy of Sciences, 3 Pasteur Str., Warsaw.

The interaction between cell surface and ingesting particle is the first step of the process of phagocytosis, after which engulfment of the particles begins. In the process of phagosome creation, the subplasmalemmal layer of cytoplasm plays an essential role. The subplasmalemmal layer of non-phagocytoting cell of free-living amoeba shows presence of the low-affinity Ca-ATPase and calcium-dependent structures /Ca-DS/. When the particle engulfment occurs, the Ca-DS are observed at the plasma membrane surrounding the cell, as well as in the submembrane layer of the emerging pseudopods. However, Ca-DS in pseudopods are visible in larger amounts than on plasma membrane of other cell regions. The low-affinity Ca-ATPase is similar to Ca-DS in its localization and distribution. In close vicinity to both of them the filamentous network can be seen.

On the other hand, the membranes of finally formed phagosomes do not show any of the above-mentioned components. In addition to the above study, in phagocytoting cells the total distribution of calcium was analysed using potassium pyroantymonate and N,N-naphthaloylhydroxylamine.

The data presented suggest that Ca-DS, Ca-ATPase and calcium, with connection to some contractile proteins, are engaged in the process of particle engulfment.

288 THE GENERATION AND THE MAINTENANCE OF CELL SURFACE POLARITY IN MDCK CELLS. K. Simons, European Molecular Biology Laboratory, Postfach 10.2209, D-6900 Heidelberg, FRG.

The plasma membrane of epithelial cells is differentiated into two domains, the apical membrane lining the epithelial lumen and the basolateral domain which faces the blood supply of the tissue. Although they are part of a continuous membrane, the two plasma membrane domains display unique protein and lipid compositions. One useful model to study epithelial cell polarity is the MDCK cell grown on permeable supports and infected with enveloped viruses. Using this system, we have shown that the newly synthesized apical hemagglutinin of influenza virus and the basolateral G protein of VSV are sorted from each other intracellularly in a single round, presumably in the trans-most subcompartment of the Golgi complex that we have named the "trans-Golgi network". Further studies have shown that also protein sorting during membrane recycling occurs with precision. Less than 0.2% of the transferrin recycling by endocytosis from the basolateral side is missorted to the apical side.

To maintain the compositional differences between the apical and the basolateral membranes, a diffusion barrier in the plane of the membrane is necessary. The domains are separated by a specific zone of cell-cell contacts which encircles the top of each cell, the tight junction. We have studied the permeability properties of the tight junctions using fluorescent phospholipids, implanted into the apical membrane by membrane fusion. The results will be presented, and on the basis of these data, a new model for tight junction structure will be discussed.

289 EPITHELIAL THYROID CELL POLARIZATION AND THE EXPRESSION OF SPECIFIC FUNCTION IN CULTURE. J. MAUCHAMP, INSERM U 270. Faculté de Médecine Nord, Bd Pierre Dramard 13326 MARSEILLE Cedex 15. FRANCE.

In culture porcine thyroid cells can form a variety of organized multicellular structures. On adhesive substrates they organize into monolayers. The basal surface is in contact with the substrate (plastic, collagen ...) whereas the apical pole is oriented towards the culture medium. In suspension the cells form polarized epithelial structures displaying inverted polarity as compared to original follicles (inside-out follicles). When only the apical surface is accessible from the culture medium iodide concentration and acute response to thyrotropin (TSH) stimulation were not expressed. When cells were cultured on permeable floating collagen gels, cells concentrate iodide and respond acutely to TSH. Inside-out follicles embedded in collagen gels undergo polarity reversal within 48h whereas specific functions (iodide trapping and organification, accumulation of thyroglobulin and sensitivity to TSH) reappeared. Culture in monolayer on the permeable bottom of culture chambers formed by a filter glued to a plastic ring allows a free access to the basal surface and the separation of apical and basal compartments. Iodide added to the basal compartment is concentrated by cells, thyroglobulin accumulates in the apical compartment and cells can be stimulated from their basal side by TSH.

Altogether these culture systems allow the study of the relations between cell polarity and expression of specific thyroid functions.

290 CONTROL OF CELL POLARITY IN AGGREGATES OF THE FRT THYROID EPITHELIAL CELL LINE. Corrado Garbi, Anna Mascia, Chiara Zurzolo, Lucio Nitsch. C.E.O.S. - Dipartimento di Biologia e Patologia Cellulare e Molecolare, II Facoltà di Medicina, Napoli, Italy.

The epithelial cell line FRT is polarized in monolayer culture but not in suspension culture of isolated cells (Eur. J. Cell Biol. 38: 57, 1985). The acquisition and stability of cell polarity was studied in FRT aggregates cultured in suspension or embedded in different types of gels.

FRT aggregates were obtained by culturing isolated FRT cells for 8 hours in suspension, on agarose coated dishes. The medium used was Coon's modified Ham's F12 containing either 5% fetal calf serum or 6 hormones. If aggregates were further cultured in suspension they originated vesicles consisting of an electron lucent lumen delimited by a monolayer of polarized cells with microvilli and tight junctions facing the culture medium. The lumen enlarged with time possibly due to directional ion and fluid transport. To directly prove the functional polarity of the cells, vesicles were infected with VSV and Sindbis virus. It was found that VSV budding was basal while Sindbis virus budding was apical, in accordance with the known budding polarity of the two viruses in FRT monolayer cultures. The cells surrounding the lumen always acquired, and maintained for months, the same structural and functional polarity, i.e. apical domain toward the culture medium. Vesicle formation from aggregates was not dependent upon RNA or protein synthesis but required the integrity of cytoskeleton components such as microtubules and microfilaments. When aggregates were embedded in collagen (100 µg/ml), gelatin (80 mg/ml), or agarose (2.5, 5 and 10 mg/ml) gels, instead of being cultured in suspension, small lumina appeared within aggregates. The cells exposed their apical domains to the lumina while the cell surfaces facing the gel were devoid of microvilli and tight junctions.

In conclusion FRT cells acquire a definite and stable polarity as a consequence of cell-cell and cell-substrate interaction. In suspension culture the cell apical domain always faces the medium. In aggregates embedded in gels the cells acquire the opposite polarity.

291 THYROGLOBULIN SYNTHESIS AND APICAL SECRETION BY THYROID CELL MONOLAYERS ON PERMEABLE SUBSTRATE. MODULATION BY THYROTROPIN. M. CHAMBARD, O. CHABAUD and J. MAUCHAMP. Inserm U.270 Faculté de Médecine Nord, boulevard P. Dramard, 13326 - MARSEILLE CEDEX 15. FRANCE

Porcine thyroid epithelial cells were cultured in monolayer on the permeable bottom of culture chambers formed by a collagen coated Millipore filter glued to a plastic ring. Cells were seeded in eagle modified Dulbecco medium without serum. The serum was added in the basal medium when the monolayer was confluent, isolating the apical compartment, inside the ring, from the basal one. We have studied with this experimental system the synthesis and the polarized secretion of thyroglobulin (Tg) and their regulation by thyrotropin (TSH). Cellular and secreted Tg were labeled by incubating the cells with 35S- methionine and quantified by immunoprecipitation with anti-porcine Tg antibodies and by densitometric analysis of autoradiograms performed after acrylamide gel electrophoresis. The following results were obtained : 1) More than 90 % of 35S-labeled Tg was secreted and accumulated in the apical medium and 70 % of apical proteins was Tg. 2) Tg synthesis and secretion were stimulated by TSH added to the basal compartment. After 48h. maximal effect (2-3 times) was achieved with 50µU/ml and stimulation was already observed after 6h. with 100µU/ml TSH. These concentrations of TSH added in the apical medium had no effect. 3) An increase in cellular Tg-mRNA level (dot-blot analysis) paralleled TSH- induced changes in Tg synthesis and secretion. 4) The volume of the apical medium decreased after chronical TSH stimulation, suggesting an apical to basal fluid transfer (40 % of initial 1ml apical volume within 48h). In this culture system apical Tg secretion and accumulation and basal TSH sensitivity were maintained as well as iodide uptake previously studied (M. CHAMBARD and coll. J. Cell. Biol. 96, 1983, 1172-1177). This system might therefore be considered as an "open follicle".

292 MORPHOLOGICAL CHARACTERIZATION OF A PUTATIVE SORTING COMPARTMENT FOR BASOLATERAL AND APICAL PLASMA MEMBRANE PROTEINS IN AN EPITHELIAL CELL LINE. Elaine Hughson, Gareth Griffiths and Kai Simons, European Molecular Biology Laboratory, Postfach 10.2209, 6900 Heidelberg, FRG.

The viral coat glycoproteins, influenza haemagglutinin (HA) and Vesicular Stomatitis Virus glycoprotein (VSV G protein), may be used as models in the study of the biosynthesis of apical and basolateral plasma membrane proteins, respectively, in polarised cells such as the Madin-Darby canine kidney (MDCK) cell line. Earlier biochemical studies at this laboratory showed that in MDCK cells at 20°C, instead of appearing on the cell surface, HA and G protein reversibly accumulated in an intracellular compartment in a terminally glycosylated form. Immunocytochemical studies, on a similar temperature block of G protein in non-polarized Baby Hamster Kidney cells, demonstrated that in these cells the viral protein accumulated in a tubular reticulum on the trans-side of the Golgi stack - the "trans-Golgi network" (TGN) (Griffiths et al., J. Cell Biol. 101, 949, 1985).

The site of sorting for membrane proteins destined for different plasma membrane domains is not yet exactly known, although the evidence points towards a late or post Golgi but intracellular site. The TGN is thus a promising candidate. In the present study we are investigating whether HA and G protein both pass through the TGN in MDCK cells. Preliminary immunocytochemical studies on ultrathin frozen sections show, in agreement with immunofluorescence results, that HA accumulates in the Golgi region at 20°C. Parallel studies with conventional electron microscopy indicate that some components of the Golgi complex are enlarged, appearing to form a tubular network. Work is in progress to characterize this structure, including the use of defined immunocytochemical markers.

293 IDENTIFICATION OF APICOLATERAL AND BASAL MEMBRANE DOMAINS IN BASAL CELLS OF MULTISTRATIFIED EPITHELIA.

Laurence Schaffar(1), Bruno A. Bernard(2), Daniel Asselineau(2), Marcelle Régnier(2), Michel Darmon(2) (1) INSERM U.210, Faculté de Médecine, Université de Nice, 06031 NICE, France, and (2) Cell Biology Department, Centre International de Recherches Dermatologiques, Sophia Antipolis, 06565 VALBONNE, France.

Monoclonal antibodies were produced in mice immunized with SV40-transformed keratinocytes. Two of them, (BC_1 and BC_4) were found to react with the plasma membrane of human basal keratinocytes. Immunofluorescence studies on frozen sections of human skin and on epidermal cell smears revealed an apicolateral distribution of the antigens recognized by BC_1 and BC_4 antibodies, while Bullous Pemphigoid (BP) antigen had a basal distribution. These antigens thus allow to define two membrane domains in basal epidermal cells, an apicolateral one in contact with neighbouring keratinocytes, and a basal one in contact with the basement membrane. By immunoprecipitation using ^{35}S-methionine and ^{3}H-mannose-labeled keratinocyte extracts, BC_1 monoclonal antibody was found to recognize a 165kD glycoprotein, while BC_4 was found not to react with blotted proteins. The corresponding antigen is probably associated with a lipid component of the plasma membrane, as shown by its detergent solubility.

These results demonstrate that basal and apicolateral topological domains can be defined in stratified epithelia in contrast with the basolateral and apical domains, yet identified in simple epithelia.

294 DEVELOPMENT AND MAINTENANCE OF FIBROBLAST MEMBRANE POLARITY

Z. Somosy, Tamara Kubasova and G. J. Köteles

"Frédéric Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene, Budapest, Hungary

The negativ charges as detected by cationized ferritine binding on primary human fibroblasts cultivated on glass substrate are polarized, i.e. they are localized mainly on apical surfaces covered by the culture medium /Somosy et al. Cell Biol. Intern. Rep. 8. 407, 1984/.
No changes can be observed in this polarity of cationized ferritine on cells embedded in collagen and it can not be influenced by the material quality of substrates. When suspending the attached cells, the binding of cationized ferritine on single cells becomes uniform. However, polarization reappears both on cells aggregated during further incubation, and on cells reattached to the substrate. In the latter case the polarized distribution of negatively charged surface sites returns to the original state.
Our results indicate that the polarity of negatively charged sites on fibroblasts are determined by cell-to-cell and cell-to-substrate contacts, similarly to the situation in epithelial cells.

295 FORMATION OF TIGHT JUNCTIONS IN INVERTED ECTODERM CELLS OF AMPHIBIA.

Tseng-Chuang Mi-pai and Zhou Mei-yun. Shanghai Institute of Cell Biology, Academia Sinica, Shanghai, China.

The phenomenon of polarization of tight junctions during the process of its formation has been reported (Tseng et al., 1986). In order to analyze the causative factor of polarization, inversion experiment was carried out. Ectoderm piece was extirpated from the early gastrula and inverted with its inner surface facing outside. By transmission electron microscopic observations different steps of tight junction formation were found at the inverted inner surface which was originally devoid of tight junctions. The relation between the tight junction polarization and the appearance and existence of the blastocoel was discussed.

296 CIRCULAR POLARITY INVOLVING SMALL NUMBERS OF CELLS IN STEM SEGMENTS OF FRAXINUS CULTURED IN VITRO AND TREATED WITH AUXIN

Z.Hejnowicz/1/,Ewa Kurczyńska/2/./1/ Institute of Agricultural Science,Agricultural Academy of Lublin,22-400 Zamość,102 H.Sawickiej,/2/ Department of Cell Biology,Silesian University,40-038 Katowice,Jagiellońska 28,Poland

The flow of auxin follows established polarity in plant cells,but if the direction of flow of auxin is changed locally polarity may be changed in a "feedback" manner. The flow of auxin in differentiating xylem is channelized in cell files and this flow is a main factor inducing vessel differentiation. Internodal segments of 1- or 2-year-old stems of Fraxinus excelsior were cut during winter dormancy. Application of auxin to the apical end induced formation of early vessels. Close to the basal end of the segment the continued basipetal flow of applied auxin is impossible. Instead of normal vessels,"rings" of vessel members are formed there. This indicates that local circular polarity has been established in groups of differentiating cells. Such rings often involve only two cells, one of which has reversed polarity. Circular polarity has been also found in intact stems, in the cambium above axillary buds. The center of this polarity is a place where an adventitious bud may be formed in disbudded stems.

297 CYTOKINESIS, CELL POLARITY, AND DIFFERENTIAL CORTICAL STIFFNESS IN A CILIATE TETRAHYMENA THERMOPHILA

Kaczanowska Janina, Bużańska Leonora & Zaleska Ewa. Institute of Zoology, Warsaw University, Krakowskie Przedmieście 24/26, 00-927/1 Warszawa, POLAND.

Ciliates manifest permanently polarized cytoskeletons. Analysis of cell surface growth, distribution of basal body landmarks and mechanical distortions of cell shapes during cell division were performed in Tetrahymena thermophila wild type, and in cells of different genotypes that failed to divide due to expression of the cdaA-1 (cell division arrest) mutation. It was found that the fission line prior to cytokinesis sharply separates the cortical areas of different stiffness. The anterior pole of the forming posterior cell is very rigid. In another experiment, distortion of the most rigid area usually appears beyond the zone of fissioning. Then this rigid cone may serve as an organizing apical center and cell changes its polarity. These data suggest that: 1/ the "spatial subdivision prior to cytokinesis" (Frankel et al., Dev. Biol. 88, 39, 1981) is related to differential stiffening of the cortex within the fission zone and 2/ that polarized arrangement of the structural elements within the cortex is monitored by the rigid apical region.

298 Light Induced Polarity of Amoeba proteus.

Krzysztof Łazowski. Nencki Institute of Experimental Biology, Polish Academy of Sciences, Department of Cell Biology, 3 Pasteur str., 02093 Warszawa, Poland

Laterally incident light induced a negative phototactic reaction of Amoeba proteus cells. The reaction is related to a semi-permanent organization of a cell. Posterior part of the illuminated cell is developed on the more irradiated side, anterior part with advancing pseudopods on the shadowed side. In order to minimize the body area exposed to light, amoeba turns its body to a parallel position in respect to a light direction, or it produces the new pseudopods on the shadowed cell side. Then, it gradually orients its body along the light direction, as the leading pseudopod advances. In order to find out the mechanisms underlying the light induced functional polarity of the amoeba the effect of different drugs on it were study. In the paper the experimental data of the effect of DMSO, digitonin and saponin on the light induced cell polarity will be presented.

299 RECENT ASPECTS OF EXOCYTOSIS REGULATION

H. Plattner, University of Konstanz, Faculty of Biology, P.O.B. 5560, D-7750 Konstanz, Fed.Rep. Germany

The following aspects make it rather difficult to establish a causal connection between the different processes occurring during exocytosis: (a) Calcium, though known to be required, regulates many processes that might or might not be relevant for exocytosis performance. (b) Other processes, like intracellular vesicle transport or exocytosis-coupled endocytosis, accompany exocytosis per se in many systems. (c) Several functional aspects may cooperate in achieving the final goal, namely the fusion of the secretory vesicle with the cell membrane.

It is therefore not surprising that many, even contradictory theories on exocytotic membrane fusion have been published (as reviewed by Plattner 1981, 1986). One major aspect still concerns the possible role of proteins in exocytosis regulation. A considerable problem resides in the fact that exocytosis is a very brief and locally very restricted event.(This could lead to overlaps and even to counteracting effects.) However, this may also represent a chance, provided one could take advantage of a synchronous and regularly structured model system.

In principle only two such systems have been found and established for exocytosis research. One is the neuromuscular junction, particularly of the frog, and the other is represented by certain ciliated protozoa, notably Paramecium. Both these systems contain preformed exocytosis sites with presumable membrane-integrated and -associated proteins. In the neuromuscular junction these are represented by the "active zones" (with rows of freeze-fracture particles and attached electron dense materials; Heuser et al. 1979). In paramecia preformed exocytosis sites contain "rosette" freeze-fracture particles and "connecting materials" between cell membrane and secretory organelles ("trichocysts");(for review, see Plattner 1986). The functional importance of these ultrastructural elements for exocytosis performance in paramecia is supported by the fact that mutations devoid of these features are incapable of exocytotic membrane fusion (Beisson et al. 1976; Pouphile et al. in press).

Evidently other secretory cells like mast cells, pancreatic acinar or chromaffin cells do not possess such salient membrane specializations. If so, could it still be that membrane-integrated and -associated proteins remain - or become - associated with exocytosis sites in a less evident manner? This is indeed what we observed with membrane intercalated particles (MIP) in chromaffin cells (Schmidt et al. 1982). (There is ample evidence for the identity of the major population of MIP with membrane integrated particles). There are also reports on the occurrence of connecting materials between chromaffin granules and the cell membrane. As to the functional aspects, microinjected anti-calmodulin antibodies showed the occurrence of calmodulin on such sites and its involvement in exocytosis has been demonstrated (Kenigsberg and Trifaró 1985).

Therefore, the situation might be quite similar in principle in quite different cells. In Paramecium cells we have also localized calmodulin precisely at potential fusion sites and we could inhibit exocytosis by antibodies (Momayezi et al. 1986). For sea urchin oocytes Steinhardt and Alderton (1982) had also found evidence along these lines. This explains some functional aspects of the occurrence of "connecting material".

Numerous papers have reported on protein phosphorylation in the course of secretory activity (see Zieseniss and Plattner 1985). There are only rare hints on de-phosphorylation processes. However, if it occurred and if it were counterbalanced by re-phosphorylation, all sorts of phenomena might overlap. We therefore considered it important to find a way for inducing synchronous exocytosis in paramecia: In response to certain polyamines they release the major part of their trichocysts (~1000) within ~1 sec (Plattner et al. 1984, 1985). Surprisingly, one particular protein of a mol. weight of 65 kD was selectively and rapidly (≤1 sec) de-phosphorylated (Zieseniss and Plattner 1985). Gilligan and Satir (1982) had caught this process by using picric acid that causes trichocyst discharge before cells are instantaneously fixed. The effect we observed was strictly dependent on the dose (of trigger or inhibitor agents) and on the morphology of the exocytosis sites described above ("rosettes" and "connecting material"). Since de-phosphorylation was rapidly reversed (10-20 sec) it would not have been recognizable in a non-synchronous system.

Since so far, protein phosphorylation had been much more analyzed and emphasized in other systems, it now remains to be seen whether paramecia behave in an aberrant way or whether in others systems phosphorylation might be due to the activation of receptors, of cytosolic proteins reversibly bound to secretory vesicles or of ion pump proteins etc., or to still other phenomena, rather than to direct fusion induction. It still remains to be seen for every system analyzed so far, what primary effect is triggered by calcium and by the calcium sensitivity-conferring protein calmodulin. However, already now it appears clear, that membrane fusion during exocytosis is regulated by proteins and finally somehow executed by lipids.

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300 Ca^{2+} HOMEOSTASIS AND SECOND MESSENGER CONTROL OF EXOCYTOSIS. J. Meldolesi[1], L.M. Vicentini[1], A. Malgaroli[1], A. Pandiella[1] and F. Di Virgilio[2], [1]Dept. Pharmacol., CNR Ctr. of Cytopharmacol., Univ. of Milano, [2]Dept. of Gen. Pathol., CNR Ctr. for the Physiology of Mitochondria, Univ. of Padova, Italy.

PC12 is a cell line derived from a rat pheochromocytoma that secretes catecholamines as well as acetylcholine. When cultured in conventional media PC12 cells exhibit a chromaffin-like phenotype, with relatively few, dense ovoidal or spherical secretion granules localized preferentially in the cytoplasmic rim immediately adjacent to the plasma membrane. These undifferentiated cells will be designated as $PC12^-$ cells. Treatment of $PC12^-$ cells with nerve growth factor induces them first (within 24 hrs) to accelerate and then to stop growing. Concomitantly, the cells enlarge (about 3X) and acquire a neuronal-like phenotype, with sprouting of neurites and establishment of mutual pseudo-synaptic contacts. In NGF-differentiated PC12 cells ($PC12^+$ cells) secretion granules are preferentially redistributed to neurite varicosites as well as terminals. Because of these properties the PC12 cell line represents an interesting paradigm of neurosecretory cells capable of undergoing differentiation towards a sympathoblast-like cell. Secretion from PC12 cells can be stimulated by the application of stimuli that cause cytosolic Ca^{2+} to raise. Depolarization by high K^+ causes the opening of voltage-dependent Ca^{2+} channel and drives therefore Ca^{2+} from the extracellular medium to the cytosol. Measurement of the free cytsolic Ca^{2+} concentration, $[Ca^{2+}]_i$, by the fluorescent indicators quin2 and fura2 revealed rapid (within 0.5 sec) rises from the resting level (0.1 μM) to maxima ranging between 0.5 and 1 μM, followed by a decrease to plateau levels between 0.3 and 0.4 uM, that were maintained for over 15 min. Analyses of these data revealed that the plateau is sustained by the persistent opening of a small fraction of the Ca^{2+} channels activated by depolarization. Inactivation of voltage-gated channels appears to be voltage and not $[Ca^{2+}]_i$ dependent because it was observed also in cells depolarized in Ca^{2+}-free media. A second procedure able to increase $[Ca^{2+}]_i$ in PC12 cells is the activation of muscarinic receptors. This activation triggers in the plasma membrane the hydrolysis of phosphatidylinositol-4,5-bisphosphate, with generation of two intracellular messengers: inositol-1,4,5-trisphosphate (IP_3), that causes redistribution of Ca^{2+} from intracellular store(s) to the cytosol; and 1,2-diacylglycerol, the physiological activator of protein kinase C. In addition to Ca^{2+} redistribution, the activation of the muscarinic receptor causes in plasmalemma the opening of a voltage-independent, receptor-operated channel. Whether the opening of the channel is also mediated by IP_3 remains to be elucidated. The $[Ca^{2+}]_i$ responses to both high K^+ and muscarinic agonists are greater in $PC12^+$ than in $PC12^-$ cells. A third treatment that causes $[Ca^{2+}]_i$ to rise is that with NGF. In this case the rise is small (from 0.1 to 0.15-0.2 μM), appears after a lag phase of approximately 1 min and is independent from the voltage-gated Ca^{2+} channels. $[Ca^{2+}]_i$ rise by K^+ depolarization is very effective in causing exocytotic secretion from PC12 cells. Secretion coincides with the rapid Ca^{2+} rise occurring immediately after depolarization, and is much less pronounced during the plateau phase. $[Ca^{2+}]_i$ rises by muscarinic receptor activation and NGF treatment are without effect on secretion, possibly because they are too small and occur at slow rate. Secretion by exocytosis can be evoked also by a group of drugs known as activators of PKC, i.e. the diacylglycerol analog 1-oleyl, 2 acetyl-glycerol (OAG) and phorbol miristate acetate (PMA). These drugs have no effect on $[Ca^{2+}]_i$. The release responses induced by PMA and OAG occur at slow rate but are persistent. They are greater in $PC12^-$ than in $PC12^+$ cells. Combination of these drugs with Ca^{2+} ionopnores (that induce $[Ca^{2+}]_i$ rises of the same size as those induced by depolarization) results in large, synergistic secretory responses. In addition to stimulating release, PKC activators have complex effects on PC12 cells. Among other things they inhibit the $[Ca^{2+}]_i$ rises induced by muscarinic agonists and depolarizing agents by causing desensitization of the receptor (i.e., uncoupling of receptor occupancy from the postreceptor event, the stimulation of polyphosphoinositide hydrolysis) and inhibition of the voltage-dependent Ca^{2+} channel. Thus, PKC effects secretion in PC12 cells at multiple sites: by direct stimulation of exocytosis and by feedback regulation of the membrane events responsible for the rise of $[Ca^{2+}]_i$.

301 STIMULUS SECRETION COUPLING IN NEUTROPHILS

Tullio Pozzan(1), P.Daniel Lew(2) and Francesco Di Virgilio(1).
(1)Institute of General Pathology ,CNR Center for the Study of the Physiology of Mitochondria,University of Padova ,Padova, Italy,and(2)Dept. of Internal Medicine ,Div. of Infectious Diseases, University of Geneva, Geneva, Switzerland.

Neutrophils play a fundamental role in host defense against invading microorganisms. These cells are particulary useful for studying the mechanism of trans membrane signaling initiated by surface receptor stimulation.A variety of agonists in fact trigger a pleiotropic cellular response which depends on the type of agonist,the intensity and duration of the stimulation.In particular,neutrophils secrete the content of their granules either into the extracellular medium or/and into the phagocytic vacuole.Selective secretion of different granule populations is another feature characteristic of neutrophils. New insights into the mechanism of stimulus secretion coupling have been gained recently by using this cell type.In this report we will briefly review the experimental evidence which indicates that in neutrophils cytosolic free Ca^{2+} concentration, polyphosphoinositide breakdown products ,GTP activated processes and protein kinase C all play a pivotal role in initiating and terminating cellular responses.We will also present a model which describes the network of activating and inhibiting pathways which may be applicable to this and other cell types.

302 **MEMBRANE COMPARTMENTS INVOLVED IN SECRETORY PATHWAY IN CULTURED PROLACTIN CELLS**

Claude TOUGARD, Renée PICART and Andrée TIXIER-VIDAL, Groupe de Neuroendocrinologie Cellulaire et Moléculaire, Collège de France, 11 Place Marcelin Berthelot, 75231 PARIS CEDEX 05

Two aspects of the secretory process have been analyzed in cultured prolactin cells using electron microscope immunocytochemistry :

1. The localization of the secretory product,
2. The identification of membrane antigens of compartments involved in the secretory pathway.

Prolactin (PRL) has been detected in rat PRL cells in culture (normal PRL cells in primary cultures and clonal tumor derived GH3 cells) in all the compartments involved in biosynthesis, transport, concentration and storage of secretory products, i.e. the rough endoplasmic reticulum (RER), the Golgi complex, small vesicles and secretory granules (1, 2).

Functional studies were performed on GH3 cells treated with thyroliberin (TRH), an hypothalamic tripeptide, which is known to stimulate the rapid release of PRL and then the PRL neosynthesis. In that conditions, a rapid decrease of the PRL content of RER and Golgi cisternae was observed within the first hour of TRH treatment. At the same time, TRH induced also the formation of numerous immunoreactive vesicles in the Golgi zone as well as beneath the plasma membrane suggesting that, besides secretory granules, small vesicles loaded with PRL may serve as carriers for hormonal release (2). Modifications in the intracellular PRL distribution concomitantly to the recently described burst of secretion induced by an acute TRH effect (within 1-2 min)(3) are actually under investigation. The delayed effect of TRH on PRL synthesis was correlated with a reloading of hormonal content of RER and Golgi cisternae after 2 hours of treatment which was prevented by simultaneous exposure to cycloheximide. Moreover, evidences were obtained in favor of the existence of two intracellular routes for PRL release using two different approaches, first monensin, a drug which perturbs the traffic of secretory product at the Golgi zone level (4) and more recently reduced temperature (20° C as compared to 37° C). In both cases basal PRL release was strongly inhibited, whereas the stimulating effect of TRH on PRL release was not prevented. This was correlated with an accumulation of immunoreactive PRL in the Golgi zone and an induction of PRL loaded vesicles in response to TRH.

Taken together these findings indicated that the Golgi zone plays a key role in PRL traffic. Thus we have localized a 135 kd Golgi antigen using a specific immunological probe (A-Golgi) (5). This revealed an immunological subcompartmentation of membrane domains in both PRL cells (6). Indeed the A-Golgi labeled medial Golgi saccules, smooth vesicles and lysosomal membrane, but not secretory granule membrane. Modifications in the distribution of the Golgi antigen following acute stimulation of PRL release by TRH treatment revealed an increased membrane traffic between the Golgi zone and the plasma membrane by the way of labeled small vesicles. Monensin treatment as well as reduced temperature did not prevent the increase in these TRH-induced small vesicles. However, no direct evidence that these Golgi derived small vesicles were loaded with PRL was yet obtained.

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303 THE ROLE OF PROTEIN KINASE C IN THE REGULATION OF STEROID SECRETION IN BOVINE ADRENOCORTICAL CELLS.

Chambaz E.M., Vilgrain I. and Hadjian A.J.

Laboratoire de Biochimie des Régulations Cellulaires Endocrines, LBIO, DRF, INSERM U 244, CNRS UA 35 01 20, Centre d'Etudes Nucléaires, B.P. 85 X, 38041 Grenoble Cédex, France.

Adrenocortical cells represent a highly differentiated system in which a cascade of specialized enzymatic activities results in the synthesis and secretion of the major corticosteroid hormones such as cortisol. These activities are under the control of hormonal effectors such as adrenocorticotropin (ACTH) and angiotensin (A II) which acutely activate cortisol production. However, while ACTH is believed to act through the increased intracellular generation of cyclic-AMP, this is not the case for A II. The possible implication of protein kinase C in the cellular response, especially with regard to A II action has been examined in bovine adrenocortical cells, with the following major observations.

(i) - Protein kinase C is present in adrenocortical cells, together with inhibitory factors and a protease activity able to generate a phospholipid independent enzyme (M kinase) from C kinase.

(ii) - The kinase is distributed between the soluble and the membrane compartments ; ACTH and A II rapidly influence this distribution, in opposite directions.

(iii) - A II, but not ACTH, induces a rapid activation of adrenocortical cell polyphosphoinositide turnover, with increased production of inositol-phosphates.

(iv) - Treatment of the cells by phospholipase C, which generates diacylglycerols elicits a marked steroidogenic effect.

(v) - Agents which are known to activate protein kinase C (diacylglycerols, active phorbols esters) mimics, in combination with Ca^{2+}, the steroidogenic action of A II.

(vi) - A potential target for protein kinase C in these cells is represented by the limiting step in steroidogenesis, i.e. cytochrome $P\text{-}450_{scc}$, specific for cholesterol side chain cleavage reaction. The purified cytochrome is phosphorylatable in vitro by protein kinase C as well as in the intact cells.

From these data, it is suggested that protein kinase C may participate in the reaction cascade leading to activated secretion by bovine adrenocortical cells under the effect of angiotensin. On the other hand, ACTH activation of cortisol secretion is thought to involve cyclic AMP and activation of c-AMP dependent protein kinase. These two pathways converging to the same cellular secretory response may influence each other by transregulation processes at different levels of the corresponding intracellular reaction cascades involved. For example, protein kinase C may be the link explaining the regulation of adrenocortical cell sensitivity to ACTH by phorbol esters and possibly by other hormonal effectors such as angiotensin.

304 FORMATION, BINDING AND ROLE OF INOSITOL-TRISPHOSPHATE IN ADRENAL GLOMERULOSA CELLS.

A. Spät(1), P. Enyedi(1), L. Hunyady(1), A. Baukal(2), G. Guillemette (2), K.J. Catt(2).
(1)Department of Physiology, Semmelweis University Medical School, H-1444 Budapest, POB 259, Hungary and (2) NIH, NICHHD, Endocrinology and Reproduction Research Branch, Bethesda, MD 20205,U.S.A.

The biological response to hormones and neurotransmitters, which bind to cell surface receptors, is generally mediated by cyclic AMP or by an increase in the concentration of cytosolic Ca2+. In 1975 Michell (1) postulated that the generation of Ca-signal is brought about by hormone-induced changes in phosphoinositide metabolism. The specific phospholipid hydrolyzed by Ca-mobilizing hormones is phosphatidylinositol-4,5,bisphosphate (PIP2) and the products of this reaction are diacylglycerol and inositol-1,4,5-trisphosphate (IP3) (2,3). Diacylglycerol activates protein kinase C (4) while IP3 induces the release of Ca2+ from a non-mitochondrial intracellular pool (3).

Aldosterone production by adrenal glomerulosa cells is physiologically controled by angiotensin II (AII), potassium ions and corticotropin (ACTH). The former two induce a rise in cytosolic Ca2+, the effect of ACTH is mediated by cAMP. This multifactorial control system renders the glomerulosa cell especially suitable for studying the formation and role of phosphoinositides in the control of cell function.

Pioneering work on glomerulosa cells (5) suggested that stimulation with AII, K+ or ACTH all enhance the synthesis of phosphoinositides, however, this phenomenon is not a primary, postreceptorial event but follows agonist-induced protein synthesis. Our first studies (6,7) showed that AII decreased the pool size of 32P-labelled phosphatidylinositol (PI) and, in turn, elicited a compensatory resynthesis of PI. These phenomena proved to be a response preceeding any Ca2+ - influx or protein synthesis and could not be evoked by K+ or ACTH. This way the response of glomerulosa cells to various stimuli corresponded to Michell's hypothesis (1).

Later studies showed that AII evokes the hydrolysis of prelabelled PIP2 and the formation of IP3 within a few seconds, ACTH and K+ have no such effects (8,9,10,). Vasopressin, which stimulates aldosterone production through V1-receptors, also enhances the breakdown of PIP2 (11). Similarly to the effect of AII in intact cells (12,13), IP3 induces Ca-release from a non-mitochondrial pool in permeabilized cells (8).

There are specific, high affinity and saturable receptors of IP3 in hepatocytes and leukocytes. The occupancy of these receptors correlates with IP3-induced Ca-release (14). In the liver, the binding sites have been found in the endoplasmic reticulum (15). In bovine adrenal cortex, where AII enhances steroid production both in the z. glomerulosa and fasciculata, we found specific and reversible microsomal binding of IP3 in both zones. The binding exhibited high affinity (Kd = 5 nM) and low capacity (0.2 pmol/mg protein) (16).

Hormone-stimulated turnover of phosphoinositides may be interrupted by lithium ions which inhibit myo-inositol-1-phosphatase. In glomerulosa cells lithium completely prevents AII-induced resynthesis of PI and also evokes a rapid decline in aldosterone production. It does not influence the steroid-stimulating effect of K+ or ACTH, indicating that the effect of lithium is specifically associated with changes in phosphoinositide metabolism (17). The inhibition of AII-induced aldosterone production may not be attributed to an impairement of steroid hydroxylation by accumulated intermediary products of the PI-cycle . Since the formation of inositol trisphosphate is not reduced within the examination period, the inhibition of the biological response may be accounted for by the formation of an isomer of IP3 (9).

In the rat adrenal cortex, where AII stimulates IP3 formation both in glomerulosa and fasciculata cells (J.F. Tait, personal communication) but only the glomerulosa responds with steroid output, in preliminary experiments we found IP3 binding sites in the z. glomerulosa alone. This finding, together with the above observations on the effect of lithium, indicates that the biological response to AII requires intact phosphoinositide metabolism and specific IP3 binding sites. Considering, however, that in spite of comparable IP3 formation the aldosterone stimulating effect of AII lasts much longer than that of vasopressin (11),we think that IP3 is a necessary but not a sufficient factor in maintaining the biological response to a Ca-mobilizing hormone.

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305 MECHANISM OF ACTIVATION OF PHOSPHOLIPASE C IN ADRENAL GLOMERULOSA CELLS

P. Enyedi, L. Hunyady, S. Kayser and A. Spät. Department of Physiology, Semmelweis University Medical School, 1444 Budapest 8.POB 259, Hungary

Hormones and neurotransmitters that inhibit adenylate cyclase (AC) usually alter the phosphoinositide and hence the calcium metabolism of the cell. The transduction of the signal to the AC involves a guanine nucleotide binding protein (Ni).The mechanism by which the receptor occupation leads to the activation of phospholipase C, i.e. to the breakdown of polyphosphoinositides is, however, unknown. Recent results suggest that GTP is involved in the process and Ni may be the coupling factor in some cells.

Angiotensin inhibits AC and stimulates phosphoinositide breakdown in adrenal glomerulosa cells. We studied whether the phosphoinositide changes are dependent on functionally intact Ni.Pertussis toxin was used in these experiments which is known to ADP-ribosylate and thereby to inactivate specifically a 41 kDa protein, the α-subnit of Ni.

Isolated cells were prepared from rat adrenal capsules and incubated for 24 hours in the presence of pertussis toxin (PT, 500 ng/ml).The treatment resulted in a full ADP-ribosylation of a 41 kDa protein, the α-subunit of Ni. Angiotensin induced an increased formation of 3H-inositol phosphates in cells prelabelled with 3H-inositol, indicating the stimulated breakdown of polyphosphoinositides. Preincubation with PT failed to influence this effect. Increased incorporation of 32P-phosphate into phosphatidylinositol was also unimpaired.

These results suggest that Ni is present in adrenal glomerulosa cells but it is not involved in the process of coupling between angiotensin receptor and phospholipase C.

306 SYNTHESIS AND SECRETION OF PLASMA LIPOPROTEINS BY A RAT HEPATOMA CELL LINE

S. Calandra, M. Ghisellini, R. Tiozzo-Costa, A. Pierangelo and R. Cingi, Istituto di Patologia Generale, Università di Modena, Via campi 287, 41100 Modena (Italy).

Liver is a major site of synthesis of plasma lipoproteins in the rat. Although in the whole animal hepatic synthesis of lipoproteins is influenced by several factors (such as diet, sex, age, and hormones) little is known on the molecular mechanisms underlying this regulation. To shed some light on this problem we studied the synthesis of apolipoproteins and the secretion of plasma lipoproteins in well differentiated rat hepatoma cells (FAO cells) which were cultured in different media. FAO cells cultured in serum free medium show the ability of secreting plasma lipoproteins, although at a low rate, for 10-12 hours. In these conditions they produce predominantly lipoproteins containing B and E apolipoproteins; they do not synthesize A-I, C and A-IV apolipoproteins. When the culture medium is supplemented with fresh rat plasma (5-20%) the synthesis and secretion of plasma lipoproteins is increased 2-3 fold. The same effect is observed if plasma is replaced by plasma fractions containing lipoproteins. Under these conditions FAO cells synthesize and secrete predominantly lipoproteins containing apolipoprotein E as the constituent peptide. It is conceivable that the lipid overload which follows the incubation of cells with lipid rich plasma induces the synthesis of apolipoprotein E as a compensatory mechanism for removing the excess of lipids present in the cytoplasm.

307 IN VIVO EFFECTS OF MONENSIN ON THE RAT THYROID

Viktor Johanson, Pyra Ring and Torsten Öfverholm, Dept. of Anatomy, University of Göteborg, Sweden

Thyroglobulin, a glycoprotein of mw 660,000, is synthesized and primarily glycosylated in the RER of the thyroid follicle cell. Secondary glycosylation takes place in the Golgi apparatus and thyroglobulin is then transferred to the follicle lumen in exocytic vesicles.

The ionophore monensin has been shown to inhibit protein transport through the Golgi apparatus in several cell systems. In this study, we have examined the ultrastructural effect of monensin in vivo on the Golgi apparatus and on the exocytic vesicles in the rat thyroid. To avoid generalized toxic effects, we have developed a method for selective administration in vivo of drugs to one rat thyroid lobe by infusion in the superior thyroid artery. The other lobe serves as control.

Monensin (5 μM) was administered for 0-2 h. The monensin-induced dilation of Golgi cisternae was studied in thyroids processed for enzyme cytochemistry for the trans cisternae markers TPPase and acPase. Dilation was seen in TPPase positive and acPase positive cisternae as well as in cisternae on both cis and trans sides of the positive cisternae. About 60 exocytic vesicle profiles were observed per cell section in control rats. This number was reduced by 40% after 30 min of monensin. Stimulation with TSH caused an almost complete disappearance of exocytic vesicles in both controls and monensin-treated rats. This is in agreement with secretion studies on isolated thyroid follicles in our laboratory, indicating that monensin does not inhibit exocytosis. Monensin greatly reduced the autoradiographic labeling of exocytic vesicles and colloid after puls-labeling with ^{3}H-leucine.

In conclusion, monensin caused dilation of several Golgi cisternae, some of which were identified as trans and GERL. The reduced number of exocytic vesicles after monensin can be explained by ongoing exocytosis combined with impeded formation of new vesicles from the Golgi apparatus. The observed reduction indicates a half-life of exocytic vesicles of the same order as calculated in previous studies in our laboratory.

308 THE SYNAPTIC VESICLE MEMBRANE PROTEIN P38 IS ALSO PRESENT IN ENDOCRINE CELLS, AND IN BOTH NEURONS AND ENDOCRINE CELLS IS SELECTIVELY ASSOCIATED WITH VESICLES DISTINCT FROM PEPTIDE-CONTAINING GRANULES.
F. Navone[1], R. Jahn[2], G. Di Gioia[1], P. Greengard[2], and P. De Camilli[1], 1) CNR Center of Cytopharm., Dept. Med. Pharm., Via Vanvitelli 32, 20129 Milano, Italy; 2) Lab. Mol. and Cell. Neurosci., The Rockefeller University, 1230 York Ave., New York, New York, 10021, USA.

P38 is a major intrinsic membrane glycoprotein of synaptic vesicles (R. Jahn et al, PNAS 82:4137-4141, 1985, and B. Wiedenmann et al, Cell 41:1017-1028, 1985). The goals of this study were to establish if P38 is present in all nerve endings, if in nerve endings it is shared by the two types of secretory organelles [small synaptic vesicles (SSV), and large dense-core vesicles (LDCV) i.e. vesicles that are known to contain peptide neurotransmitters], and if it is also present in secretory organelles of non-neuronal cells. To do so we studied the localization of P38 immunoreactivity in a variety of tissue and brain regions by quantitative immunochemistry and by light and EM immunocytochemistry. We have found that P38 is present in all nerve endings and that, in addition, it is also present in a large number of (and possibly in all) peptide-secreting endocrine cells. By EM immunocytochemistry, P38 in nerve endings was found to be selectively associated with SSV and absent from LDCV. A similar selective association with SSV was previously reported for Synapsin I, a peripheral protein of the membrane of synaptic vesicles. In endocrine cells, P38 was found to be localized on smooth surfaced vesicles of variable size and with clear content scattered throughout the cytoplasm but more concentrated in the trans-Golgi region. Little, if any, P38 was associated with the membrane of peptide-containing secretory granules. Our results support the idea that SSV of neurons are organelles of a secretory pathway distinct from the regulated pathway involved in peptide secretion. Moreover, they suggest that an endomembrane system biochemically related to SSV of neurons is present also in endocrine cells. Since we have previously shown that endocrine cells do not contain Synapsin I, this protein might be involved in the adaptation of this endomembrane system to neuronal secretion.

309 SECRETORY GRANULES FROM BOVINE NEUROHYPOPHYSIS CONTAIN AN ENDOPEPTIDASE WHICH CLEAVES SYNTHETIC PRO-OCYTOCIN PEPTIDE AT LYS-ARG DOUBLET.
M. Camier, C. Clamagirand, H. Boussetta, C. Fahy, A. Morel, M. Rholam, P. Nicolas, P. Cohen. Groupe de Neurobiochimie Cellulaire et Moléculaire, Université Pierre et Marie Curie, UA 554 du CNRS, 96 boulevard Raspail, 75006 PARIS, France.

In the bovine hypothalamus, the nonapeptide ocytocin (OT) derives from the processing of a precursor (pro-OT-Np) in which the NH_2-terminal OT is connected to 10,000 Mr neurophysin (Np) by the Gly-Lys-Arg processing sequence.

In order to characterize the processing enzymes a synthetic octacosapeptide which reproduces the 1-17 sequence of the precursor, i.e. OT-Gly^{10}-Lys^{11}-Arg^{12}-$(Tyr\text{-}NH_2)^6$(1-5)Np was prepared and used as substrate together with various synthetic peptides corresponding to its NH_2- and COOH-terminal sequences. Both substrates and reaction products were identified by HPLC, TLC, NH_2-terminal sequencing and "flight time" mass spectrometry. Lysates of highly purified neurohypophysial secretory granules, free of lysosomial contamination, were used as source of proteases. A 58 Kda endopeptidase activity was detected together with both amino- and carboxypeptidase B-like activities. The endopeptidase activity cleaves the peptide bond on the carboxyl side of the Lys-Arg doublet and so, releases both ocytocin-Gly^{10}-Lys^{11}-Arg^{12} and $(Tyr\text{-}NH_2)^6$(1-5)Np. The functional properties of this endopeptidase are discussed with particular emphasis to i) the specificity towards the Lys-Arg doublet ii) the role of the conformation around the basic pair as studied with synthetic analogs. It is hypothesized that in combination with the amidating enzyme detected in granules this endoprotease and the carboxypeptidase B-like enzyme might be involved in the "in vivo" processing of the OT-Np precursor.

310 CATIONIC FERRITIN UPTAKE BY CULTURED HYPOPHYSEAL CELLS TREATED WITH A PROTEINASE INHIBITOR, BOC-DPhe-Phe-Lys-H.
Gy. Gaál, E. Bácsy, G.B. Makara, Gy. Rappay. Institute of Experimental Medicine, Hungarian Academy of Sciences, H-1450 Budapest, POB 67, Hungary

BOC-DPhe-Phe-Lys-H, a tripeptide aldehyde has been shown to decrease in vitro prolactin secretion by hypophyseal cells to one fourth of the control value. Growth hormone secretion of the same cultures decreased to one third as compared to controls (Rappay et al., Life Sci. 36: 549, 1985).

It has been suspected that the drug exerts its inhibitory effect primarily at the cell membrane. Therefore, the membrane turnover in the presence of 10^{-4} M BOC-DPhe--Phe-Lys-H of prolactin and growth hormone cells was investigated with cationic ferritin added to the incubation medium either during or after treatment with the drug.

The cationic ferritin given simultaneously with the drug or after a two-hour treatment of the cells did not affect prolactin secretion. Electron microscopy revealed that in the presence of 10^{-4} M BOC-DPhe-Phe-Lys-H the cytoarchitecture of the hypophyseal cells remained unchanged. The cationic ferritin particles in control cells were localized mainly at contact sites between two neighbouring cells and occasionally also in lysosome-like structures as early as after five minutes. After thirty minutes, ferritin particles were often found in crinophagic vacuoles in addition to small vesicles near the cell membrane of the parenchymal cells. The treatment with the tripeptide aldehyde did not alter the distribution of cationic ferritin around the cells and the intracellular pathway of ferritin seemed to be unaffected.

311 ULTRACYTOCHEMICAL LOCALIZATION OF X-PROLYL-DIPEPTIDYL(AMINO)PEPTIDASE IN *pep4-3* MUTANT OF *Saccharomyces cerevisiae* REVEALED A NEW CLASS OF MICROCONVEYORS FOR (GLYCO)PROTEINS EXOCYTOSED BY YEAST. *Jos. Vořišek* (1), Ladka Kalachová (1), Vl. Starý (1) and Kalju Vanatalu (2). (1) Institute of Microbiology, Czechoslovak Academy of Sciences, CS-14220 Praha, Vídeňská 1083. (2) Institute of Chemical Physics and Biophysics, Estonian Academy of Sciences, SU-200026 Tallinn, P.O.Box 670.

The secretory path for yeast (glyco)proteins starts in the endoplasmic reticulum (ER) and continues via Golgi compartment into the membrane bound vesicles that fuse with the plasmalemma and open in the periplasmic space (Schekman, Trends Biochem Sci 7,243-249, 1982). Our study of peptidase mutant of *S.cerevisiae* (*pep4-3*) revealed accumulated membranes of ER and globular microcompartments (diameter 20-50 nm), both containing the X-prolyl-dipeptidyl(amino)peptidase (DPP) reaction product and cytochemically detectable amounts of polysaccharides. Both cytochemical markers were observed also in the periplasmic space. The matrix of sectioned microcompartments - microglobules (MG) was filled with the DPP reaction product; when this was absent, it was possible to distinguish a single line boundary (seen also on freeze etched replicas) connected by the coat of fibrillar spokes to the cytoplasmic matrix so that the diameter of the whole coated MG reached 50-100 nm. The MG with DPP activity were observed in the cytoplasmic matrix (disperesed in single cells or grouped in the growth apex of budding ones), in the nucleoli and in mitochondria. From the results was deduced the role of MG in the secretory path of yeast. The adjoined statistical calculations of binominal distributions explain why the MG were not distinguished in previous cytologic studies of wild strains of yeasts.

312 CALCIUM AND ß-ADRENERGIC RECEPTOR REGULATION OF ^{3}H-PROTEIN SECRETION IN RAT PAROTID: CYTOCHALASIN D INHIBITION. Claire Huleux(1), C.Dreux(1), S.Busson(2), A.M.Guérin(3), L. Ovtracht(2), G.Herman(1), V.Imhoff(1) and B.Rossignol(1). (1)Biochimie des transports cellulaires, CNRS UA 1116, Université Paris-Sud, 91405 Orsay Cédex,France. (2)Centre de Cytologie expérimentale, CNRS, 67,rue M.Günsbourg, 94200 Ivry-sur-Seine,France. (3)INSERM U 282, Hôpital H. Mondor, 94010 Créteil,France.

In rat parotid gland, ^{3}H-protein secretion is strongly stimulated by Isoproterenol (IP) and Forskolin (FK) (which both induce cAMP accumulation). Carbachol (Cch) and A23187 (which induce calcium movements) only caused a small protein release. Cytochalasin D (CD), which disturbs the microfilament system, strongly and immediately inhibited the secretory response to IP or FK. CD rapidly induced the formation of big empty vacuoles in the unstimulated acinar cells. Under IP stimulation in the presence of CD, only few secretory granules (SG) remained (like in IP stimulated cells), but the vacuoles were filled with labeled secretory products. The inhibition of the ß-adrenergic secretory response by CD could be due to an intracellular discharge in the vacuoles instead of an extracellular discharge. CD did not inhibit the cAMP level increase induced by IP or FK. But CD inhibited by 50 % the Ca^{2+} efflux (independent of cAMP) stimulated by IP, when it had no effect on Cch induced Ca^{2+} efflux. In the presence of Ca^{2+} in the external medium, the inhibition of the ß-adrenergic secretion by CD was partialy suppressed by addition of Cch or A23187 which promote Ca^{2+} entry in the cell. From these results, it could be suggested that CD inhibits ^{3}H-protein secretion throughout two mechanisms : intracellular ^{3}H-protein discharge and inhibition of Ca^{2+} movements induced by the activation of the ß-adrenergic receptor.

313 EFFECT OF ISOPRENALINE ON HAMSTER SEMINAL VESICLE SECRETORY CELLS. PRELIMINARY RESULTS. Christiane Arnold and Lucinda R. Mata. Department of Cell Biology, Gulbenkian Institute of Science, Oeiras

In the secretory cells of castrated hamster seminal vesicle, apical secretory granule volume and number are increased as a result of more reduced exocytosis than secretory protein production(1). This work is aimed to test the capability of castrated hamster seminal vesicle secretory cells to respond to the stimulation of exocytosis.

Castrated and normal hamster seminal vesicles were removed and processed for electron microscopy 2 hours after isoprenaline (IPR) or saline injection.The morphometric study focused on the amount of membrane in the cell apex and in the secretory vacuoles, as well as on the amount of secretory products in apical and Golgi zone. After IPR treatment, the relative increase of apical membrane is higher in the castrated than in the normal animal. The decrease in the relative volume of apical secretory granules is higher in the castrated animal than in the normal one whereas the numerical density of those granules is unchanged in the castrated but increases in the normal hamster. The relative number of Golgi secretory granules decreases in the castrated animal as in the normal one but the decrease in relative volume of those granules is higher in the castrated than in the normal hamster. This work shows that IPR stimulates both exocytosis and secretory granule migration from the Golgi to the apical zone.The relatively higher increase of exocytosis induced by IPR in the castrated animal is related to the previous accumulation of apical secretory granules.

These results allow the conclusion that the capability of hamster seminal vesicle secretory cells to respond to the stimulation of exocytosis by IPR is maintained after castration and are consistent with a possible testosterone-dependent process of secretory product discharge in the normal situation.

(1) Mata,L.R. & David-Ferreira,J.F. - Biol.Cell, 53, 165-177, (1985).

314 A PARTICULAR PROCESS OF ACCUMULATION OF SECRETORY PRODUCT ELABORATED BY THE GRANULAR ENDOPLASMIC RETICULUM.

Constantin Crăciun, Dept.Biology, Univ.Cluj-Napoca, R-3400, Cluj-Napoca, Romania.

The ultrastructural mechanisms of the formation and evolution of a particular cellular constituent named by us eracosome (endoplasmic reticulum accumulation body) is identified and described in the glandular cells of the vas deferens epithelium of Porcellio scaber Latr. (Isopoda, Crustacaea).

Initially, in the proximal vicinity of a Golgi complex there appears a small accumulation of an electrondense material, around a few GER cisternae. This formation grows gradually both by deposition of the Golgi substance and especially of the proteic substance broght in by GER cisternae. The formation increases in diameter through incorporation of more and more GER cisternae into its mass. Once entered, the cisternae loose their ribosomes and become tubular (cca. 90 nm in diameter), but, at the same time, they retain continuity with the GER cisternae of the surrounding cytoplasm. At this stage, the eracosome is a multitubular oval-spherical formation of 3 - 5 µm in diameter. The proteic material synthesized in GER cisternae is transported directly through their lumens into the tubules found in the mass of eracosome. This material concentrates and electrondensifies at the interior of each tubule into 7 longitudinal bands (10 - 12 nm in diameter, each). By resorbtion of the tubule walls, from the center toward the periphery of the eracosome, this changes finally into a huge secretory granule (4 - 5 µm in diameter) which will be eliminated from the cell by exocytosis.

315 DISSOCIATION OF THE MEMBRANE CONDUCTANCE INCREASE AND CORTICAL GRANULE EXOCYTOSIS DURING ACTIVATION OF XENOPUS EGGS.

Michel Charbonneau and Dennis Webb. Laboratoire de Cytologie Expérimentale, Université de Rennes I, Rennes, France.

Monospermy is ensured in the **Xenopus** egg by two distinct mechanisms : 1) The fertilization potential -a transient membrane depolarization due to an increase in the Cl^- conductance. 2) The cortical reaction -an exocytosis of granules situated in the egg cortex leading to elevation and modification of the vitelline envelope. The study of the interrelationship between these two events is complicated by their close temporal association. We have recently succeeded in recording the membrane conductance change in the absence of the cortical reaction. Treatment of **Xenopus** eggs with either the weak bases NH_4or procaine, or with the lectins, WGA or SBA, prior to activation, prevented both the exocytosis and vitelline envelope elevation. However, the membrane conductance increase could still be triggered either electrically or by the Ca^{2+} - ionophore A23187 or by sperm. Each of the lectins modified the form of the membrane conductance change in a specific way while in general it was prolonged by the weak bases. At the ultrastructural level, cortical granules were displaced away from the plasma membrane by weak bases but not by lectins. Weak bases also dramatically altered the organization of the microvilli. The similar effect of weak bases at high extracellular pH suggest that their mode of action involves a rise in intracellular pH. The two lectins appear to exert their effect in a different way. The exocytotic modification of the plasma membrane may influence the membrane conductance changes involved in the fertilization potential.

316 POTASSIUM-INDUCED EXOCYTOSIS IN MAST CELL.

Anna Németh (1), Pál Magyar (2). (1) Laboratory I of Electron Microscopy, Semmelweis University of Medicine, Budapest H-1450, Tüzoltó u. 58,(2) Department of Pulmonology Semmelweis University of Medicine, Budapest 1125 Diós árok 1/c, Hungary

Potassium chloride has been found useful in diagnosis of bronchial asthma. The question arises wether K^+ has a direct effect on mast cells. This paper reports on the effect of K^+ on rat peritoneal mast cells by following exocytosis (degranulation) morphologically and measuring histamine release. K^+ was found to induce exocytosis and histamine release from 10 to 200 mM, the most efficient concentration being 150 mM. The degranulating effect of K^+ was completed within one minute at 22°C. When comparing the effect of other monovalent cations, Rb^+ and Cs^+, similarly to K^+, were found to induce exocytosis and histamine release from mast cells, Cs^+ being less effective than Rb^+. Na^+ and Li^+ did not exert any effect on mast cell exocytosis. Antihistamine drugs (Intal, Ketotifen) inhibited K^+-induced exocytosis of mast cells. However in comparison to 48/80-initiated degranulation, the inhibition obtained with K^+-induced degranulation was less efficient.

317 INDUCTION OF THE ACROSOME REACTION OF BULL SPERMATOZOA BY OUTER ELECTRICAL FIELDS.

R. Petzoldt (1), T. Klemke (2). (1) Academy of Sciences of GDR, DDR-1115 Berlin, Roessle-Str. 10, and (2) Section of Biology, Humboldt University, DDR-1040 Berlin, Invalidenstr. 43, GDR.

Capacitation and acrosome reaction are basic cellular processes of the sperm cells in mammalia. The mechanisms of acrosome reaction are at present objects of an intensive discussion. In accordance to the in vivo conditions, mainly chemical signals are discussed to induce acrosome reaction. In this paper is shown, that the acrosome reaction is significantly increased due to the application of an outer electrical field to a suspension of bull spermatozoa.

The calculated electrical field strength of 20 us pulses causing an electrical breakdown is in the range of 3 - 4 kV/cm in the chamber. By modification of the incubation conditions, the amount of spermatozoa with acrosome reaction is changed. After a preincubation in bovine serum albumin (BSA) -Tyrodesolution, the part of membrane-intact sperms with acrosome reaction is increased. Using BSA in the incubation medium an increasing amount of head-to-head agglutinated spermatozoa in observed. Comparing the fresh ejaculated and reactivated deep frozen bull sperm cells the behaviour is similar in regard to the effect of the electrical field on the acrosome reaction. The stimulation of the motility and a higher degree of viability of the deep frozen spermatozoa in the case of low electrical fields is surprising.

The results are discussed from the point of electrical breakdown and fusion of the cell membrane and acrosome membrane.

318 IMPAIRED AROMATASE ACTIVITIES OF THE PLACENTAL MICROSOMES PREPARED FROM DIABETIC PREGNANCIES - Jenő Egyed, Dept.of Obstet.and Gynaec.,Postgreduate Medical School, H-1135 Budapest, Szabolcs u. 35, Hungary

In vitro aromatase activities as regulators of placental estrogen production were investigated in normal and diabetic pregnancies.Fresh placentae were treated and microsome fractions were obtained as described by Gibb et al./1980/. The final sediments were resuspended in phosphate buffer and stored in small portions at -20°C. Enzyme activities were tested measuring the 3-sulphatase activity in incubations with 3-H-dehydroepiandrosterone sulphate /DHAS/ as substrate /details were described earlier /Egyed and Oakey, 1985/ and, under the same conditions the aromatase activities were determined by 3-H testosterone substrate /as was published by Gibb.

Results could be summarized as listed below:

1. The 3-sulphatase activities were normal /in the range of 100-200 pmol DHA /30 min x mg enzyme protein/ and thete was no difference between normal and diabetic cases
2. Contrary, there was found a significant difference in the aromatase activity between normal /30-40% conversion of testosterone to estradiol/40 min x mg enzyme protein/ and diabetic placentae /below 5% conversion/. It means that the enzyme activities were significantly decreased in cases of normoglycaemic diabetic patients while in normal cases the aromatase activities were remained.

Analyzing these data an evidence is raising about a "diabetic-type stereochemical alteration" of the microsomal protein synthesis as a possible explanation for the earlier controversal data in this field.

319 MORPHOBIOCHEMICAL STUDIES ON ADRENALINE SECRETION IN GUINEA PIG'S ADRENAL MEDULLA IN EXPERIMENTAL ANAPHYLAXIS. Bogumiż L. Kmieč. Department of Histology and Embryology, IBM, Medical School, 90-136 Lodz, Narutowicza 60, Poland.

As a result of the performed investigations a 50% loss of adrenaline (A) from adrenal medullae in experimental anaphylaxis was observed - which is fully confirmed by ultrastructural investigations of this gland. In submicroscopic pictures epinephrocytes showed all modes of A secretion known so far; moreover, in experimental anaphylaxis characteristic pictures of secretion of this catecholamine, not described so far, were noticed. This mode was called a "massive and facilitated" exocytosis. The mechanism of secretion in this case is the formation, from granular vesicle membranes, of characteristic canaliculi and cisternae enabling a very fast secretion of so great amounts of A by their connexions with the epinephrocyte cellular membrane. This mechanism is correlated and confirmed by submicroscopic pictures of freeze-fracturing and cryofracturing replicas. This main mechanism of secretion was confirmed by submicroscopic pictures of chromaffin and uranaffin ultrahistochemical reactions as well. The uranaffin reaction was found in adequate correlation with a decrease·of amount of ATP only in the homogenates of adrenal glands as compared with amounts of other investigated adenine nucleotides. Decrease of activity of DBH and characteristic changes in activity of total LDH and its isoenzymes in adrenal medulla studied by means of histochemical methods confirm above mentioned observations too. Increase of amounts of Ach in adrenal homogenates, and not changed activity of AchE may be connected with significant increasing in concentration of Ca^{2+} in epinephrocytes and with characteristic function of ATP-ases responsible, in author's opinion, for "massive and facilitated" secretion of A in anaphylaxis.

320 INTRACELLULAR AND INTERCELLULAR DISTRIBUTION OF SECRETORY PRODUCT IN THE SUBCOMMISSURAL ORGAN. W. Lösecke (1), W. Naumann, G. Hoheisel, and G. Sterba (2). (1) Institute of Neurobiology and Brain Research, Academy of Sciences of the GDR, Magdeburg, and (2) Department of Cell Biology and Regulation, Section of Biosciences, Karl Marx University, Leipzig, German Democratic Republic.

To analyze the secretory process of the subcommissural organ (SCO) of the rabbit indirect immuno-metal cytochemistry (protein A-gold technique) was applied to ultrathin sections of low-temperature-embedded tissue. By means of a rabbit antiserum against bovine Reissner's fibre (RF) and particulate gold marker solution, in both the ependymal and hypendymal SCO-cells two substructures display a positive immunocytochemical reaction: (a) sacs, which originate from the rough endoplasmic reticulum, and (b) vacuoles, which are pinched off from the Golgi complex. The secretory product of the ependymal SCO-cells is discharged apically into the cerebrospinal fluid where it becomes condensed to RF ("ventricular" route of secretion). In the hypendymal zone, the secretion is released into cavity-like intercellular spaces and distributed, thereafter, throughout an extracellular channel system including (i) dilated intercellular spaces, (ii) branching basal lamina labyrinths, and (iii) distinct perivascular spaces. All these compartments are thought to function in secretion directed towards hypendymal blood vessels ("basal" route of secretion).

321 VASOACTIVE INTESTINAL PEPTIDE (VIP) STIMULATES ALDOSTERONE SECRETION BY RAT ADRENALS IN VIVO. G.G.Nussdorfer, Giuseppina Mazzocchi, Claudia Robba. Department of Anatomy, University of Padua, Padua, Italy.

Recent investigations demonstrated the existence of VIP-ergic fibers in the rat zona glomerulosa (Holzwarth, J.Auton.Nervous System. 11:269,1984), but studies so far available failed to show any effect of VIP on aldosterone secretion by isolated rat adrenocortical cells (Enyedi et al., Acta Physiol.Hung. 61: 77,1983). Male rats were sc infused (using osmotic Alzet micropumps) for 7 days with dexamethasone (0.25 mg/kg.day) or dexamethasone plus ACTH (2 IU/kg.day). Other animals were given captopril (10 mg/kg.day) or captopril plus angiotensin II (AII)(0.2 mg/kg.day). Thirty min. before the sacrifice, half of the rats in each group received a single ip injection of 0.1 mg/kg VIP. Long-term treatment with dexamethasone significantly decreased the plasma level of corticosterone and aldosterone, whereas prolonged captopril administration lowered only that of aldosterone. ACTH and AII infusions completely reversed the effects of dexamethasone and captopril, respectively. VIP did not provoke any significant change in the blood concentration of corticosterone, but significantly raised aldosterone plasma level in both dexamethasone/ACTH- (76%) and captopril/AII-treated (36%) animals. VIP was able to enhance significantly aldosterone secretion in captopril-treated rats (38%), but not in dexamethasone administered animals. These results indicate that VIP specifically stimulates rat zona glomerulosa in vivo, and that this effect is not mediated by the interference of VIP with the hypothalamo-hypophyseal axis and the renin-angiotensin system. Moreover, our findings suggest that a normal level of circulating ACTH is required in order to VIP can exert its acute adrenoglomerulotropic action, whereas a normal rate of endogenous synthesis of AII is not needed.

322 LONG-TERM EFFECTS OF 4-AMINOPYRAZOLO-PYRIMIDINE (4-APP) ON THE ZONA FASCICULATA OF DEXAMETHASONE/ACTH-TREATED RATS. Piera Rebuffat, Giuseppina Mazzocchi, G.G.Nussdorfer. Department of Anatomy, University of Padua, Padua, Italy.

Male rats were sc infused for 14 days (using osmotic Alzet micropumps) with dexamethasone (0.25 mg/kg day) and ACTH (2 IU/kg.day). On the 7th day, some animals received daily ip injections of 5 mg/kg 4-APP, for 7 consecutive days. Part of 4-APP administered rats were fed a high (5%) cholesterol diet. 4-APP treatment significantly decreased both plasma (66%) and intra-adrenal total cholesterol (78%), while did not affect plasma corticosterone level. This treatment provoked a significant increase in the average volume of zona fasciculata cells (28%), which was exclusively due to smooth endoplasmic reticulum (SER) proliferation. The volume of the lipid droplet compartment was notably reduced (68%), whereas that of DAB-positive bodies (peroxisomes) displayed a 3-fold increase. High cholesterol diet completely reversed the effects of 4-APP. The morphological changes induced by 4-APP are interpreted as the morphological counterpart of the activation of adrenocortical endogenous synthesis of cholesterol, which in the rats is very low: in fact, in the SER and also in peroxisomes is located the 3-hydroxy-3-methylglutaryl coenzyme A reductase (the rate limiting enzyme of cholesterol synthesis) and in the lipid droplets is stored exogenous cholesterol taken up from serum lipoproteins. This compensatory response, enabling zona fasciculata cells to maintain a normal level of hormonal output in the absence of a normal supply of exogenous cholesterol, is conceivably independent of the activation of the hypothalamo-hypophyseal axis, since it occurs in rats treated with dexamethasone, which suppresses ACTH release, and ACTH, at a dosage ensuring the normal maintenance of the adrenal growth.

323 EFFECTS OF ETHINYL ESTRADIOL ON THE RAT ADRENAL CORTEX

M.M.Magalhães (1), M.C.Magalhães (1), C.Hipólito-Reis (2), Teresa A.M.Serra (3) and M.Lurdes Gomes (1). (1) Institute of Histology and Embryology and (2) Laboratory of Biochemistry - Faculty of Medicine of Oporto, (1) Center of Experimental Morphology and (3) Center of Experimental Cytology - University of Oporto, 4200 Porto, Portugal.

The ultrastructural and biochemical alterations produced by an hypocholesterolemic substance (17 α-ethinyl estradiol) on the rat adrenal cortex were studied. Thirtheen male rats, 2 months old and ~200g in weight were injected subcutaneously with ethinyl estradiol (10 mg/Kg/day) during 9 days;rats injected in the same way but with propylene glycol were used as controls. The animals were sacrificed on the 10th day and the adrenals from some of them were processed for electron microscopy. The adrenal from the remaining rats were used for measurement of the gland cholesterol and corticosterone, which were also measured in the blood.

In estradiol-treated rats the zona fasciculata cells presented numerous cytoplasmic processes, increase in the size of mitochondria and decrease in the number of lipid droplets. The quantitative analysis showed an increase of the volumetric density of mitochondria and cytoplasmic processes and a volumetric decrease of the lipid droplets in the treated rats, when compared with normal ones.In treated rats the levels of cholesterol and corticosterone in gland and in blood were significantly decreased when compared with the values of the control animals.

These data are highly suggestive that: 1) The normal rat adrenal corticosteroidogenesis depends of the cholesterol delivery from plasma;2) In our experimental conditions,the cholesterol and corticosterone concentrations in the adrenal, and the endoplasmic reticulum fine structure are not compatible with the adrenal "de novo" cholesterol biosynthesis.

324 CORRELATION OF THE MORPHOFUNCTIONAL DIFFERENTIATION OF THE HUMAN FETAL HYPOTHALAMUS, THE HYPOPHYSIS AND THE ADRENAL CORTEX.

L.V. Kuznetsova(1), A.T. Gyévai(2), V.I. Altuchova (1), B. Bukulya (2), V.A. Schachlamov(1). (1) Institute of Human Morphology, Academy of Medical Sciences USSR, Moscow, Curupy 3, and (2) Institute of Experimental Medicine, Hungarian Academy of Sciences, H-1450 Budapest, POB 67, Hungary

In previous reports, we have demonstrated that immunopositive ACTH-producing cells and immunoreactive ACTH in the pituitary of human fetus can be detected as early as 6-7-week of pregnancy (1,2). However, immunoreactive ACTH in the fetal blood appeared only at the 12th week of pregnancy. The aim of the present study was to follow the activity of 3-beta-hydroxysteroid dehydrogenase in the fetal adrenal glands during ontogenesis, and to measure the content of cortisol in the fetal blood as a function of gestational age. From our data we conclude that a. the 3-beta-hydroxysteroid dehydrogenase activity and cortisol secretion increase as a function of gestational age. The latter data are in good agreement with our earlier results in rat embryos (3). b. the fetal zone is not directly involved in cortisol secretion. c. the fact that cortisol concentration in fetal blood increase during fetal life and reaches a maximum at parturition is in contrast to the ACTH concentration which falls during this period. This phenomenon suggests that changes in ACTH-producing activity of the fetal pituitary at the end of pregnancy are not matched by similar changes of cortisol secretion by the fetal adrenal glands.

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325 VITELLOGENIN HORMONAL CONTROL IN THE GREEN FROG, RANA ESCULENTA

A.M. Polzonetti-Magni (1), A. Gobbetti (1), M. Zerani (1), O. Carnevali (1), V. Botte (2). (1) Department of Cellular Biology, University of Camerino, Italy and (2) Institute and Museum of Zoology, University of Naples, Italy.

In most oviparous Vertebrates, vitellogenic maturation of the developing oocyte is largely dependent upon availability of the suitable yolk precursor, vitellogenin, in the serum. It has now been proved that vitellogenin serum level results from a balance between rate of the synthesis in the liver and that of uptake in the ovary. In all non-mammalian Vertebrates so far studied, synthesis and uptake of vitellogenin have both been proved to be hormone-controlled processes. The synthesis of vitellogenin was induced by estradiol 17β in male Rana esculenta. The native vitellogenin has a molecular weight of about 400.000 daltons but the analysis in polyacrylamide gels in the presence of sodium dodecyl sulfate proves a molecular weight of about 200.000. This suggests that the native state of Rana vitellogenin has dimeric structure, like in Xenopus. Furthermore, the vitellogenin titre was measured in the plasma of Rana esculenta during annual cycle by rocket immunoelectrophoresis in 1% agarose gels. The results are consistent with the experimental model in proving that an appearance of vitellogenin in the serum precedes in time or at most coincides with ovarian recovery. The differences in the temporal relationship between rise in the vitellogenin titre and initiation of ovarian growth point to the existence of independent control of these two processes. In fact estradiol is known to enhance the hepatic synthesis of vitellogenin; gonadotropins, on the other hand enhance both synthesis and uptake. More recent evidences, however, tend to underline that other hormones may effect vitellogenin stimulation in the liver, at least by playing a permissive role. Our re-

sults show that hypophysectomy in Rana esculenta impairs synthesis of vitellogenin when induced by "in vivo" administration of estradiol. Gonadotropins enhance the uptake, presumably by acting directly on the oocyte plasma membrane. In addition, our data support a direct pituitary intervention on liver synthesis and/or release of vitellogenin. Hormonal response tends to increase from November to July. This could be the expression of a modification, throughout the sexual cycle, of liver sensitivity to the hormones.

326 TARGETING OF PROTEINS TO AND INSERTION INTO MEMBRANES. Bernhard Dobberstein, Joachim Lipp and Marie-Therese Haeuptle, European Molecular Biology Laboratory, Postfach 10.2209, D-6900 Heidelberg, FRG.

Eucaryotic cells are organized by several membrane-surrounded compartments of which each contains a characteristic set of proteins. Most of these proteins are synthesized on polysomes in the cytoplasm and are then transported to their respective site of residence. Many aspects of this protein topogenesis have become clear in recent years by the work of several groups. The most important steps can be summarized as follows:

Protein localization requires specific signal sequence and receptors that recognize them. The signals are relatively small and they target the protein to the respective membrane by a ligand-receptor mechanism. How the protein is then translocated across a membrane is not understood. Proteins might stay at their site of membrane insertion or they are sorted and transported to other destinations. Secretory- and membrane proteins for instance undergo intracellular transport and sorting.

Signal sequences can not only target a protein to a particular membrane, they can also, in conjunction with other topogenic sequences, determine the location and orientation of a protein in the membrane. We will report on sequences that determine the orientation of proteins in the ER membrane.

327 **TARGETING OF PROTEINS TO AND TRANSPORT ACROSS THE MEMBRANE OF THE ENDOPLASMIC RETICULUM (ER).** Jonathan A. Rothblatt and David I. Meyer, European Molecular Biology Laboratory, D-6900 Heidelberg, Federal Republic of Germany

Nascent secretory proteins and integral membrane proteins (of organelles comprising the secretory pathway) possess an N-terminal, or infrequently an internal, stretch of hydrophobic amino acids — the signal sequence. This segment of 15-30 amino acids provides information necessary for the specific targeting of the ribosomal complex to the membrane of the endoplasmic reticulum (ER) and the subsequent vectorial translocation of the polypeptide across or into the ER membrane. Soon after its emergence from the ribosome the signal sequence interacts with a cytosolic factor, the Signal Recognition Particle (SRP). Directed association of the translational machinery with the ER is facilitated through the interaction of SRP with its receptor in the rough ER — the Docking Protein. Integral proteins of the rough ER responsible for binding of the ribosome and translocating the nascent chain remain unidentified. Modifications of the polypeptide, such as signal sequence cleavage and asparagine-linked glycosylation, occur on the lumenal aspect of the rough ER.

The yeast Saccharomyces cerevisiae possesses all of the complex pathways for protein and membrane traffic of higher eukaryotic species, yet is relatively simple to manipulate at the genetic level. The potential for utilizing the yeast system to genetically dissect protein translocation lead us to develop a cell-free assay of translocation by yeast rough ER. A homologous cell-free system has been derived from S. cerevisiae that allows the translation, translocation and glycosylation of yeast mating factor α and invertase. The precursors were translated in a yeast lysate from mRNA obtained by in vitro transcription of the MFα1 and SUC2 genes. Inclusion of yeast microsomes resulted in the glycosylation of the α-factor precursor, which was demonstrated to be sequestered within the membrane vesicles. Similar results, including signal sequence cleavage, were observed for invertase. Processing of secretory proteins translated in a yeast lysate could not be achieved using microsomes derived from canine pancreas, nor were yeast microsomes active in a wheat germ translation system.

Investigation of the temporal relationship between the translation of prepro-α-factor and its translocation across the membrane of the microsomal vesicle showed that the translocation and glycosylation of this protein can be uncoupled from its translation. Such posttranslational processing is dependent upon the presence of ATP in the assay system. It is not, however, affected by a variety of uncouplers or ionophores, including CCCP, valinomycin, nigericin, DNP, KCN, or N-ethylmaleimide. This mechanism of translocation is significant as it indicates that a protein of 18.6 kD is capable of crossing a membrane posttranslationally. For the moment, this phenomenon seems to be restricted to prepro-α-factor in the yeast cell-free system. Neither invertase nor IgG-κ light chain could be translocated posttranslationally in yeast, nor was such processing observed for α-factor in the wheat germ system supplemented with canine pancreatic microsomes.

328 SIGNALS THAT DIRECT PROTEINS TO DISTINCT INTRAMITOCHONDRIAL LOCATIONS.

E.C. Hurt, A.P.G.M. van Loon and D. Roise. Biocenter of the University, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

Most polypeptides of internal mitochondrial compartments are synthesized in the cytoplasm as larger precursor polypeptides with amino-terminal presequences. Translocation of these precursors across one or the two mitochondrial membranes is energy-dependent and accompanied by proteolytic removal of the presequence (Schatz and Butow (1982), Cell 32, 316-318). In contrast, polypeptides of the mitochondrial outer membrane lack transient presequences. Their insertion into the outer membrane is specific, does not require an energized mitochondrial membrane (Freitag et al. (1982), Eur. J. Biochem. 126, 197-202; Gasser et al. (1983), J. Biol. Chem. 258, 3427-3430) and is mediated by permanently attached amino-terminal sequences (Hase et al. (1984), EMBO J. 3, 3157-3164).

Employing gene fusion, we tested the following two questions: (a) are presequences sufficient to direct an attached protein to and into mitochondria? (b) do presequences have information for directing a protein to its correct intramitochondrial location? The presequences of subunit IV of yeast cytochrome c oxidase (an inner membrane protein exposed to the matrix), of cytochrome c_1 (an inner membrane protein exposed to the intermembrane space) and mitochondrial alcohol dehydrogenase (ADHIII, a matrix enzyme) were fused to the amino-terminus of dihydrofolate reductase (DHFR), a cytoplasmic enzyme from mouse. All three presequences directed the attached DHFR into yeast mitochondria: they contain information for intracellular targeting. The presequences of subunit IV and of ADHIII targeted the mouse DHFR into the mitochondrial matrix whereas the presequence of cytochrome c_1 directed the dihydrofolate reductase into the intermembrane space: presequences also contain informaton for correct intramitochondrial sorting. The first half of the cytochrome c_1 presequence or the first twelve amino acids of the 70kd protein of the outer membrane show characteristics of "matrix-targeting" sequences and can function as such: they transport attached DHFR into the matrix. Thus, each of the four proteins studied contains a "matrix-targeting" sequence at its amino terminus and this sequence is followed in the case of cytochrome c_1 and of the 70kd protein by a stretch of amino acids probably functioning as a "stop-transfer" sequence for one of the two mitochondrial membranes. Our experiments suggest that intramitochondrial sorting may be determined by combination of "matrix-targeting" and "stop-transfer" sequences at the amino-terminus of the imported polypeptide itself. How do presequences find mitochondria? Peptides consisting of part or all of the presequence of subunit IV of cytochrome c oxidase have been chemically synthesized and tested for their interaction with membranes. Each of the peptides, although quite soluble in aqueous solution, spontaneously insert into lipid membranes as measured by various physico-chemical methods and can uncouple mitochondria. The amphipathic properties of presequence peptides are consistent with a model in which the presequence of subunit IV forms an amphiphilic alpha-helix.

329 THE ROLE OF THE TRANSIT PEPTIDE IN THE IMPORT OF PROTEINS BY CHLOROPLASTS

Luis Herrera-Estrella, Marc Van Montagu and Guido Van Den Broeck
Genetics Department
State University of Gent,
Ledeganckstraat 35, B-9000 Gent
Belgium

In plant cells, most chloroplast proteins are encoded in the nuclear DNA and are the products of protein synthesis on cytoplasmic ribosomes, many as soluble precursors, which are then translocated through the plastid envelope membranes, processed and assembled into their final organellar compartment.

We have studied the function of the transit peptide of the precursors of a light harvesting chlorophyll a/b protein and of a small subunit of the ribulose bisphosphate carboxylase. Although both proteins are imported by chloroplasts their final organellar destination is different. We have found that both transit peptides mediate the translocation and processing of heterologous proteins into chloroplasts. However, the final organellar destination of an imported protein seems to be determined by the nature of the protein itself and not by the origin of the transit peptide used to mediate chloroplast import.

330 SIGNALS FOR MICROBODY UPTAKE IN THE GLYCOLYTIC ENZYMES OF TRYPANOSOMES

B.Swinkels[1], W.Gibson[1], K.Osinga[1], P.Borst[1], P.Michels[2], O.Misset[2], F.Opperdoes[2], R.Wierenga[3] and W.Hol[3]. [1]Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands, [2]International Institute of Cellular and Molecular Pathology, Avenue Hippocrate 74, B-1200 Brussels, Belgium, [3]State University of Groningen, Nijenborgh 16, 9747 AG Groningen, The Netherlands.

The parasitic protozoa grouped in the family Trypanosomatidae are characterized by an unusual microbody, called the glycosome, which contains the major part of the glycolytic pathway in addition to (parts of) several other metabolic pathways. The transfer of glycolysis from its usual location in the cytosol to a microbody allows a study of the changes in cytosolic glycolytic enzymes required to redirect them into a microbody. We expect these changes to provide information on the mechanism of entry of proteins into microbodies (peroxisomes, glyoxysomes) in general.

We isolated and studied the Trypanosoma brucei genes for glyceraldehyde-P dehydrogenase (GAPDH), P-glycerate kinase (PGK), triose-P isomerase (TIM) and aldolase. T.brucei contains one gene for TIM, and two completely identical linked genes for both GAPDH and aldolase. Three tandemly linked genes were found for PGK, two of which encode the cytosolic and glycosomal isoenzymes respectively. The product of the third gene, which is characterized by an insertion of 100 amino acids, has not yet been identified. The amino acid sequences, predicted by these glycolytic genes, share 45-55 % homology with their counterparts in other organisms.

The cytosolic and glycosomal PGKs are 93 % homologous at the amino acid level. The differences found in the amino acid sequences are of two types: the glycosomal PGK has a C-terminal extension of 20 amino acids, not present in the cytosolic isoenzyme or in any eukaryotic PGK; the glycosomal PGK has gained 14 positive charges over the cytosolic enzyme. These results establish that few modifications are required to convert a cytosolic enzyme into a microbody enzyme. Either of these differences or a combination of both must determine the difference in the routing of the cytosolic and the glycosomal PGK.

Signals for uptake into glycosomes should also be present on other glycosomal enzymes. None of the three other glycosomal enzymes, GAPDH, TIM and aldolase, has a significant C-terminal extension. We also do not see an analogous sequence internally in positions where the T.brucei enzymes differ from their counterparts in other organisms. The only obvious feature that is shared by the glycosomal enzymes analysed, is a high net positive charge; higher than any of their counterparts in other organisms studied thusfar. Inspection of the 3-D structure of some of these glycolytic enzymes shows the spatial organization of the extra positive charges: the glycosomal PGK, TIM and GAPDH all contain two clusters of positive charges which are at equal distance in all three enzymes. We propose these two clusters and their spatial organization to be the signal for microbody uptake and we have set out to test this in an in vitro import system.

Reference: Osinga et al., 1985, EMBO J., 4, 3811-3817.

331 **SEQUENCE REQUIREMENTS FOR NUCLEAR LOCATION OF PROTEINS.** Richardson, W.D.,[1] Roberts, B.L.[2], Kalderon, D.[3], Colledge, W.H.[4], Smith, A.E.[2], and Dingwall, C.[5] 1. Zoology Department, University College London, Gower St., London WC1E 6BT. 2. Integrated Genetics, 31 New York Avenue, Framingham, Mass. 01701, USA. 3. Department of Biochemistry, University of California, Berkeley, Ca 94720, USA. 4. National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA. 5. CRC Molecular Embryology Group, Zoology Department, Downing Street, Cambridge, CB2 3EJ, England.

Nuclear proteins are synthesised in the cytoplasm and subsequently migrate to the nucleus, but little is known about how proteins enter and accumulate in nuclei (see ref. 1 for review). During most of the cell cycle, the nuclear contents are partitioned from the remainder of the cell by a double membrane known as the nuclear envelope. Embedded in the envelope are nuclear "pore complexes", doughnut-shaped structures with a central channel about 9nm diameter. The pores appear to act as a molecular sieve, allowing unhindered passage of proteins smaller than about 15kd, but retarding entry of larger proteins in a size-dependent manner up to an exclusion limit of 60-70kd. Rapid entry of large proteins into the nucleus therefore requires a mechanism other than passive diffusion, and one possibility is that the pore aperture dilates in response to a specific structural feature of large nuclear proteins. Since proteins isolated from nuclei and injected into the cytoplasm reaccumulate in the nucleus, the "signal" which specifies nuclear accumulation must be an integral part of the mature protein.

By genetic manipulation of coding sequences, microinjection of the DNA into cultured fibroblasts, and visualisation of the accumulated proteins by immunofluorescence microscopy, we have identified nuclear location signals in a variety of nuclear proteins. The signals are short (less than 10 amino acids) and contain a predominance of basic residues. The best characterised signal to date derives from SV40 large-T. It consists of the string of amino acids pro lys lys^{128} lys arg lys val, of which the integrity of lys-128 is of crucial importance (2,3). This sequence is essential for nuclear accumulation of SV40 large-T (2,3), and is sufficient to redirect the cytoplasmic enzyme pyruvate kinase (PK) to the nucleus when incorporated into its structure (4). Mutational analysis of the SV40 signal, and comparison with those of other proteins including polyoma virus large-T (5), adenovirus 72kd DNA-binding protein, and SV40 capsid proteins VP1 and VP2 (6), leads to the conclusion that shape and charge rather than precise amino acid sequence may be of primary importance for signal recognition.

Some of the nuclear location signals we have identified fall in internal regions of the protein, while others are found at the amino- or carboxy-terminus. We have investigated the positional requirements further by inserting a single copy of the SV40 large-T signal at one of six different locations in the 529 amino acid sequence of PK, each corresponding to a convenient restriction site in the coding sequence. Five of the six hybrid proteins accumulated in nuclei. Thus, the SV40 large-T signal is functional when inserted after PK amino acids 17, 218, 295, 417 or 522, but not when inserted after residue 232. Inspection of the X-ray structure of PK (7) reveals that the functional sites occur close to the termini or in loops connecting two structural motifs (α-helix or β-sheet), all of which are exposed in the overall structure of the protein. The inactive site lies in an internal, unexposed region. There appears therefore to be little constraint on the placement of the nuclear location signal except that it may need to be accessible on the surface of the native protein. Indeed, a given protein may contain more than one signal, and polyoma virus large-T provides an example of this (5).

Little is known about how nuclear accumulation of SV40 large-T is achieved, and the experiments described do not directly address this question. Binding to DNA or to the cellular protein p53 does not seem to be involved, since cytoplasmic mutants of SV40 large-T retain these activities (8). More is known about the nuclear protein nucleoplasmin. This 33kd protein enters the nucleus as a pentamer (9), through nuclear pores (10). A 12kd subdomain of the protein is required for entry but not retention in nuclei, a single 12kd "tail" sufficing for transport of each pentamer. A complete nucleoplasmin cDNA has now been cloned and sequenced (C.D., in preparation), and we are now attempting to determine the nature of the signal in the "tail", by constructing and expressing nucleoplasmin-PK fusion vectors. Several regions of high local basicity, typical of the nuclear location signals described above, occur within the nucleoplasmin "tail". These experiments may therefore provide a test of the hypothesis that nuclear location signals of the type first identified in SV40 large-T specify unidirectional transport through nuclear pores.

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332 LARGE DOMAINS OF SECRETORY AND MEMBRANE PROTEINS CAN BE COTRANSLATIONALLY TRANSLOCATED ACROSS ER MEMBRANES. Marie-Theres Haeuptle, Joachim Lipp, Ibrahim Ibrahimi and Bernhard Dobberstein, European Molecular Biology Laboratory, Postfach 10.2209, 6900 Heidelberg, FRG.

How secretory and membrane proteins are translocated across ER membranes is not yet understood. In the wheat germ cell-free system signal recognition particle (SRP) has been found to arrest secretory polypeptide chain elongation when approximately 70 aa have been translated (Meyer et al., Nature 297,647, 1982). Upon interaction with docking protein (DP), an integral membrane protein of rER, the elongation arrest is released. From these and other data it was interpreted that proteins were translocated in an extended manner and possibly threaded through the membrane amino acid by amino acid.

We expressed secretory and membrane proteins in an in vitro transcription-translation-translocation system. When SRP-arrested translation products were analyzed by SDS-PAGE, we observed a ladder of nascent peptides the size of which increased with prolonged time of incubation of the translation mixture. Upon addition of rough microsomes, the peptides could be chased into mature proteins sequestered in the membrane vesicles. Thus, polypeptides pre-synthesized up to 3/4 of the precursor size remain translocation competent. We are currently investigating whether chain elongation is required for the translocation of such pre-synthesized domains. In another approach, we analyzed the translocation capacity of a secretory protein truncated by different means at its C-terminus. When early stop codons were introduced into the mRNA sequence, an 85 aa pre-peptide was efficiently processed but not a 60 aa chain. Truncated polypeptides were also synthesized either from mRNAs run-off transcribed from restriction enzyme-cut plasmids or by translation in the presence of oligonucleotides complementary to defined internal mRNA sequences. The thus shortened pre-protein was poorly translocated and processed, even when it was still 103 aa long. We therefore suggest that a certain size domain must form before translocation of pre-secretory proteins across ER membranes can successfully be initiated.

333 THE TRANSLOCATION SIGNAL OF THE HUMAN TRANSFERRIN RECEPTOR. M. Zerial, P. Melançon, C. Schneider and H. Garoff. European Molecular Biology Laboratory, Postfach 10.2209, 6900 Heidelberg, F.R.G.

In eucaryotic cells most secretory, plasma membrane and lysosomal proteins contain a cleavable N-terminal signal peptide which targets the polysome to the membrane of the endoplasmic reticulum (ER), with the aid of a signal recognition particle (SRP). However, there are a number of examples of proteins which are inserted across the ER membrane but do not have a cleavable N-terminal signal sequence. These include the human transferrin receptor (TR). TR is a plasma membrane glycoprotein which has its N-terminus oriented towards the cytoplasm, whereas the bulk of the protein, including the C-terminus, is on the lumenal (or extracellular) side of the membrane. In order to test whether this molecule contains a translocation signal within its transmembrane segment, we fused the 5' part of the TR cDNA which codes for the N-terminal part of the TR, including the membrane spanning peptide, with each of the cloned cDNAs encoding for mouse dihydrofolate reductase (DHFR) and chimpanzee α-globin. The cDNA hybrids were inserted into a SP6 based transcription vector (pGEM-2) and transcribed into capped RNA for the purpose of in vitro translation in the presence of RER microsomes. Using a protease assay, we were able to demonstrate that the TR/DHFR and TR/α-globin hybrids, produced in our in vitro system, were both inserted across the ER membrane. The insertion was shown to be SRP dependent. In contrast, neither DHFR nor α-globin could be translocated. Finally, in a second engineering experiment, we could limit the "signal sequence" of the TR precisely to the transmembrane region.

The major conclusion of this work is that the transmembrane region of the human TR acts both as membrane anchor and signal sequence, targeting the polysome to the ER membrane. This process, as in the case of proteins with cleavable signal sequence, occurs via SRP.

334 PRESENCE OF EXPOSED MANNOSE-6-PHOSPHATE ON PIG THYROGLOBULIN.
Wolfgang Neumüller, Volker Herzog. Department of Cell Biology, Goethestr. 33, D-8000 München 2, West-Germany

Pig thyroglobulin (TG) is a phosphorylated glycoprotein which carries 3 phosphate groups per monomer. Digestion experiments revealed that 1 of the 3 phosphate residues is bound to the (Endoglycosidase H sensitive) high-mannose carbohydrate side-chain. The second phosphate group is linked to the (Endoglycosidase D sensitive) complex carbohydrate side-chain, while the third appears to be attached to an amino-acid residue, since it is not susceptible to cleavage by any of the endo- and exoglycosidases tested.

1 of the 3 phosphate groups is particularly sensitive to digestion with alkaline phosphatase and is, therefore, exposed on the surface of the TG molecule. As Endo-H cleaves no additional phosphate from TG after treatment with alkaline phosphatase, the exposed phosphate group appears to be part of the high-mannose carbohydrate side-chain. This suggests its presence as phosphorylated mannose. In order to test this hypothesis, TG was incubated with α-mannosidase which resulted in the removal of up to 1 phosphate molecule. In order to test for the presence of mannose-6-phosphate (man-6-P), an enzymatic assay was applied to H_2SO_4-hydrolysates of TG. The results unequivocally show that the man-6-P recognition marker is a constituent of the pig TG molecule.

TG is the predominant exportable protein of thyroid follicle cells. The presence of exposed man-6-P indicates, therefore, that TG does not follow the usual intracellular man-6-P receptor-mediated transport pathway from the endoplasmic reticulum to lysosomes and suggests that another signal may disrupt this routing mechanism in thyroid follicle cells. (Supported by Deutsche Forschungsgemeinschaft).

335 SORTING OF TRANSFERRIN AND ASIALOGLYCOPROTEIN DURING RECEPTOR MEDIATED ENDOCYTOSIS IN HEPG2 CELLS. W. Stoorvogel, and Ger J. Strous, Department of Cell Biology, University of Utrecht, Medical School, Nic.Beetsstraat 22, 3511 HG Utrecht, The Netherlands

HepG2 cells internalize both transferrin and asialoglycoproteins via receptor mediated endocytosis. These ligands are endocytosed by the same coated pits and vesicles. Therefore, they must be separated intracellularly, as (apo)transferrin is recycled back to the cell surface together with its receptor (the transferrin receptor), while asialoglycoprotein is not returning to the cell surface, but is uncoupled from its receptor (the asialoglycoprotein receptor) and degraded. A conjugate of asialoorosomucoid (ASOR) and horseradish peroxidase (HRP) was prepared. Its binding to the asialoglycoprotein receptor was fully comparable to the binding of ASOR to this receptor. Endocytosis of 125I-ASOR/HRP was followed by cell fractionation on Percoll gradients. When the cell homogenate, after binding and interiorization of the labeled complex, was incubated with diaminobenzidine (DAB) and peroxide prior to centrifugation, a density shift due to HRP induced polymerization of DAB in ASOR/HRP-containing compartments was observed. Co-labeling of cells with 131I-transferrin showed initially this label present in the same compartment as 125I-ASOR/HRP, since gradient fractions containing radioactive transferrin exhibit the same change in density as vesicles containing the ASOR/HRP complexes. HepG2 cells were incubated at 0°C in the presence of 125I-ASOR/HRP and 131I-transferrin for 60 min. After 10 min incubation at 37°C in the presence of 0.3 mM primaquine (a weak base, causing neutralization of acidic compartments) 100% of the endocytosed transferrin was localized in ASOR/HRP containing vesicles (100% co-shift). After incubation at 37°C without primaquine for 2, 5, and 10 min, sorting was $< 50\%$, $> 50\%$, and complete, respectively. At 23°C internalization and sorting are slowed down. After 30 min at 23°C only a minor portion of internalized transferrin was sorted from the ASOR/HRP. Control experiments showed that an ASOR/HRP induced shift of transferrin is completely specific and dependent on receptor binding and internalization of ASOR/HRP.

336 THE BIOSYNTHESIS AND TARGETING OF LYSOSOMAL HYDROLASES AND OTHER PROTEINS

Bryan G. Winchester
Department of Biochemistry, King's College (University of London), Kensington Campus, Campden Hill Road, London W8 7AH, U.K.

Precursors of lysosomal hydrolases are synthesised on ribosomes associated with the rough endoplasmic reticulum. The nascent proteins are transported across the membrane and into the lumen of the endoplasmic reticulum after interaction of a hydrophobic N-terminal signal sequence with a signal recognition particle. The en-bloc transfer of a preformed oligosaccharide from a lipid carrier, dolichyl pyrophosphate, to asparagine residues also occurs co-translationally. The signal peptide is removed and the processing of the asparagine-linked oligosaccharide begins in the endoplasmic reticulum. The glycosylated pro-enzymes are then transferred by a vesicular process to the Golgi complex, where the lysosomal proteins are segregated from other glycoproteins by the acquisition of a lysosomal recognition marker, mannose-6-phosphate. The phosphorylated pro-enzymes bind to a high affinity mannose-6-phosphate receptor. The ligand receptor complex leaves the Golgi via a coated vesicle to reach a pre-lysosomal compartment, which has an acidic pH that causes dissociation of the pro-enzyme and receptor. Further proteolytic processing is initiated before the lysosomal enzymes are sequestered in vesicles to form primary lysosomes, in which the final maturation occurs. Other pathways not involving the mannose-6-phosphate receptor are responsible for the transport of lysosomal membrane-associated hydrolases and, in at least some cells, the soluble lysosomal hydrolases. The biochemical and microscopical investigations of the signals and location of the steps in these pathways and the origin of other lysosomal proteins will be reviewed.

337 CHARACTERISTICS AND FUNCTIONAL CAPACITY OF LYSOSOMAL PROTEASES.

Heidrun Kirschke, Institute of Biochemistry, Medical Faculty, Martin-Luther-University, DDR-4020 Halle (Saale), PO Box 184, GDR.

Lysosomes contain a rich selection of proteolytic enzymes necessary for the complete degradation of proteins. Protein breakdown mediated by lysosomal enzymes is presumed to be initiated by endopeptidases (proteinases) which are rate limiting. The process is continued by lysosomal exopeptidases which are extremely active in the degradation of even quite large peptides. Many dipeptides, as well as amino acids, can diffuse through the lysosomal membrane for final hydrolysis by dipeptidases elsewhere in the cell.

Only cysteine and aspartic proteinases have been detected in lysosomes (other than specialized organelles of granulocytes and spermatozoa). The catalytic mechanism of the cysteine and aspartic proteinases allow them to act at the acidic pH of the lysosomal contents, whereas the serine and metallo-proteinases seldom show much activity below pH 7.

Amongst the lysosomal cysteine proteinases cathepsin B (EC 3.4.22.1), cathepsin L (EC 3.4.22.15) and cathepsin H (EC 3.4.22.16) have so far been the most investigated endopeptidases. They are widely distributed in mammalian tissues. The amino acid sequences and other characteristics show them to belong to the same superfamily of proteinases as papain and other plant cysteine proteinases.

Cathepsin D (EC 3.4.23.5) is the only well characterized lysosomal aspartic proteinase. It has been isolated from and estimated in so many mammalian species and tissues that one can assume its ubiquitous distribution. The lysosomal aspartic proteinases cathepsin D and cathepsin E belong to the same superfamily of proteinases that depend on the carboxyl groups of two aspartic acid residues for their catalytic activities as for instance the pepsins, renin and some fungal proteinases.

The lysosomal proteinases have been characterized under in vitro conditions and it is appropriate to consider the relevance of such information to an understanding of the proteolytic functions of lysosomes. There is no doubt that the interior of the lysosomal system provides conditions that are close to those that have been found to be optimal for most of the enzymes: an acidic pH, and the presence of low molecular weight thiol compounds as activators of the thiol-dependent proteases. In one important respect the conditions of the in vitro experiments are different from the interior of the lysosomal system. The effective concentrations of the enzymes in lysosomes are perhaps 10 000-fold greater than those used for biochemical studies, and moreover, the enzymes are present together, acting synergistically. That means that the specificity for peptide bond cleavage is really much broader than it would appear to be in the usual test systems, and the rates of hydrolysis are enormously greater. It follows from this that estimates of the proteolytic capacity of the lysosomal proteinases deduced from in vitro studies to in vivo conditions are usually too low.

338 THE ENDOCYTIC COMPARTMENT. BIOCHEMISTRY AND ROLE IN INTRACELLULAR ACIDIFICATION. W.H. Evans. National Institute for Medical Research, London, U.K.

Membrane vesicles derived from various parts of the endocytic compartment of liver were prepared and characterized. This compartment plays a central role in regulating intracellular membrane trafficking. Ligand-receptor complexes transferred to the endocytic compartment from the plasma membrane are dissociated and processed separately, involving functional interactions with lysosomes and, via a return route, various domains of the plasma membrane (1).

Acidification in the endocytic compartment of isolated hepatocytes was demonstrated by using fluorescently-labelled asialoglycoproteins (2). These studies showed that ligands remained intact in this compartment where the pH is 5-6. Transfer to lysosomes, occuring by warming the cells to 37 , was rapid and led to ligand degradation. However, some ligands, e.g. asialotransferrin, were not degraded but were returned to the plasma membrane.

No distinctive enzymes were identified in endocytic membranes (3). A monensin activated Mg ATPase of high specific activity in conjunction with the presence of undegraded radio ligands,provided a marker for endocytic membrane vesicles. Endocytic vesicles, purified on Nycodenz density gradients, acidified when Mg-ATP was added, and the process was reversed by addition of monensin (4). An analysis of the protein, glycoprotein and lipid composition indicated that hepatic endocytic membranes resembled more closely plasma membranes than other membranes and organelles (5). These results suggest that the membranes comprising the endocytic compartment may be regarded as an invaginating "intracellular" domain of the plasma membrane when the cell is better able to control the endocytic uptake and processing of a variety of ligands.

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339 AUTOPHAGY AND ITS ROLE IN THE BREAKDOWN OF CELLULAR COMPONENTS

J.Kovács, A.L.Kovács, G.Rèz, M.Sass. Dept. of Gen. Zoology, ELTE Univ.,H-1088 Budapest, Puskin u. 3., Hungary

Autophagy is a major pathway for the degradation of cellular components in animal cells. Two variants of this process can be distinguished by their different morphology and sensitivities to different factors: the macroautophagy and the microautophagy. The former is a multistep process which begins with the sequestration of parts of the cytoplasm by smooth-surfaced membranes. Autophagosomes thus formed gain hydrolytic enzymes in a second step by fusion with lysosomes which is then followed by the degradation of the sequestered substances. Microautophagy is a less well characterized process during which small bits of cytoplasm are directly taken up by lysosomes.

Autophagic vacuoles deriving from macroautophagy can be encountered in many cells during normal steady state conditions and are extremely abundant in starved and/or injuried cells. This increase may reflect the increased rate of sequestration and/or decreased rate of degradation. Therefore, the enlargement of the autophagic compartment by itself is not a reliable indicator of the stimulation of intracellular breakdown. Recent advances in the quantitation of the autophagic process provide solution for this problem. Correlated morphometric evaluation of changes in the autophagic/lysosomal compartment and of the intracellular proteolysis by biochemical methods in perfused liver (6), in isolated liver cells (3) and in cultured fibroblasts (5) revealed close correlation of autophagy to proteolysis:increased breakdown was always accompanied by enlargement of the autophagic compartment. Examination of the kinetics of sequestration of cytoplasmic enzymes into AVs led to the conclusion that the rate of this process is proportional to the measured rates of proteolysis (2).

New methods were developed for the study of kinetic parameters of the autophagic process. The rate of sequestration can now be estimated by introducing labeled sucrose into the cells and measuring its accumulation in sedimentable organelles (1). In isolated liver cells the sequestration measured

by this method proceeds at a rate of about 5%/hour. (The rate of protein degradation may be as high as. 4-5% in these cells.)

In our laboratory the turnover of AVs was estimated by morphometry from the time course of regression of AVs following the administration of cycloheximide. This drug is a potent suppressor of autophagic sequestration in many types of cells. We observed rapid regression of AVs in the pancreatic acinar and seminal vesicle cells of mice pretreated with Triton X100 and in seminal vesicle cells pretreated with estron acetate. The average half-life of AVs was about 6-8 min in both types of cells and was independent of the size of autophagic compartment (the cytoplasmic volume fraction of AVs was about 1% and 0.5% in pancreatic and seminal vesicle cells, respectively after a 4 h treatment with Triton X100 and about 0.25% in estrogen treated seminal vesicle cells). Similar data were obtained earlier for the regression of AVs in liver cells using a mixture of amino acids and/or insulin as suppressors of sequestration (4,6). Therefore, we think that the capability for processig AVs with a half-life of about 6-10 min may be a common property of mouse and rat cells. This uniformity of the turnover of AVs lends support to the long proposed view that autophagy is regulated mainly by controlling the rate of segregation.

We investigated the effects of two known inhibitors of proteolysis leupeptin and vinblastine on the turnover of AVs. We observed rapid decay of AVs and a half-life of about 6 min in the liver, pancreas and seminal vesicle cells of mice pretreated with leupeptin. However, the volume fraction of dense bodies enlarged enormously after this treatment. These data indicate that the AVs rapidly fused with lysosomes but their further processing (degradation) was inhibited. On the other hand,AVs accumulated in great number and their regression was very slow in the cells of mice pretreated with vinblastine. This may be explained by supposing that the drug impaired the fusion between the autophagosomes and lysosomes. Indeed, purified AV fractions isolated from mouse pancreas treated by vinblastine for different lengths of times exhibited decreasing lytic capacities as the enlargement of AV compartment proceeded. However, this may be the consequence of heavy overload of the lysosomal compartment by the substrate due to stimulated sequestration.

Taken together, the availability of the above methods seems to permit the reliable estimation of the amount of cytoplasmic material involved in autophagy and the velocity by which the sequestered components undergo disintegration in the cells under different experimental conditions. It also seems possible to determine the site of actions of various agents influencing cellular autophagy.

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340 INTRACELLULAR PROTEIN CATABOLISM: STUDIES BY MICROINJECTION AND TRANSPLANTATION TECHNIQUES. R. John Mayer, M. Gaskell, R. Earl, E. Billett, H. Mangiapane, F. Doherty. Department of Biochemistry, University of Nottingham, Nottingham NG7 2UH, U.K.

Microinjected glycolytic enzymes [^{125}I] are rapidly segregated into the nuclear-intermediate filament cytoskeleton in confluent 3T3-L1 cells and are subsequently degraded at a slow rate ($t_{\frac{1}{2}}$ 124-165 h) by a lysosomal mechanism. Microinjected underivitized lactate dehydrogenase is similarly segregated into the nuclear-intermediate cytoskeleton. Lactate dehydrogenase is released from the nuclear-intermediate filament fraction by 2-mercaptoethanol in the presence of SDS. Similarly, approximately 50% of Sendai virus HN and F glycoproteins [^{125}I] introduced into growing HTC cell plasma by reconstituted Sendai virus envelope (RSVE)-target cell fusion are segregated into the nuclear-intermediate filament cytoskeleton and degraded at a slow rate ($t_{\frac{1}{2}}$ 90 h) by a lysosomal mechanism. In contrast microinjected bovine serum albumin and haemoglobin in 3T3-L1 cells are generally not found in the nuclear-intermediate filament fraction and are degraded in the cytosol by non-lysosomal (acidotropic-independent) mechanisms. Microinjected in vitro translated polypeptides are distributed in cytosol, membranes, actin filaments and nuclear-intermediate filament fraction in growing 3T3-L1 cells and are interestingly subjected to little or no degradation. In confluent cells the microinjected polypeptides are found in recipient cell cytosol and intermediate filament fraction but are again not degraded. Endogenous [^{35}S]-radiolabelled and non-radiolabelled proteins accumulate in the nuclear-intermediate filament cytoskeleton of 3T3-L1 cells in the presence of leupeptin. The data therefore suggest that some endogenous and exogenous proteins can be deposited in an insoluble form associated with the nuclear-cytoskeleton. This insoluble protein may be a cellular site for protein that cannot be immediately degraded or may be one intermediate step in the normal lysosomal degradative pathway.

341 MICROINJECTION STUDIES ON SELECTIVE.PROTEIN DEGRADATION RELATIONSHIPS BETWEEN STABILITY, STRUCTURE, AND INTRACELLULAR LOCATION.

Scott W. Rogers and Martin C. Rechsteiner. Department of Biology, University of Utah, Salt Lake City, Utah 84112 USA.

Cells degrade their proteins with remarkable specificity. We have used two approaches to study the relationship between the structure of a protein and its intracellular stability. First, over 48 proteins for which the primary and x-ray structures are known were radiolabeled and introduced into HeLa cells by RBC-mediated microinjection. We then measured the half-life and intracellular location of each protein. Analysis of these data has shown that the role of lysosomes in degradation of injected proteins is minor and neoselective, and that the subcellular location of a protein following injection may have a significant influence on its stability. Furthermore, no correlations were found between stability and protein size, isoelectric point, hydrophobicity, or thermostability of an injected protein may be related to an interplay between its location and the presence of unstable amino acids in disordered surface regions.

Second, we have examined the primary sequences of 12 proteins with intracellular half-lives less than 2 hours. Each rapidly degraded protein contains at least one region of 12 to 45 amino acids rich in proline, glutamic acid, serine, and threonine (PEST) which is flanked by clusters containing positively charged amino acids. PEST regions may also be indentified by features common in their hydropathy plots. Similar inspection of 400 more stable, structurally characterized proteins revealed that only three contained weak PEST regions. On the basis of this information, we anticipated that caseins, which contain several PEST sequences, would be rapidly degraded within eucaryotic cells. This expectation was confirmed by RBC-mediated microinjection of 125I-caseins into HeLa cells where they exhibited half-lifes less than 2 hours. Therefore, the presence of PEST regions can confer rapid intracellular degradation on proteins containing them.

342 UBIQUITIN-PROTEIN CONJUGATES, INTRACELLULAR PROTEOLYSIS AND HEAT SHOCK RESPONSE IN MAMMALIAN CELLS

Richard G. Kulka, Hadas A. Parag and Bilha Raboy. Department of Biological Chemistry, Institute of Life Sciences, The Hebrew University of Jerusalem, 91904 Jerusalem, Israel.

Ubiquitin is a highly conserved 76 amino acid residue protein which is abundant in eukaryotic cells from yeast to man. Ubiquitin is involved in a number of basic cellular functions that include ATP-dependent intracellular proteolysis and the heat shock response. A common denominator of all the known functions of ubiquitin is its covalent linkage to other proteins. Surprisingly, little is known about naturally-occurring conjugates of ubiquitin with cellular proteins.

We have studied naturally-occurring ubiquitin conjugates in cultured rat hepatoma (HTC) cells by permeabilization and immunoblotting. ^{125}I-Ubiquitin introduced into permeabilized cells rapidly forms conjugates with endogenous proteins. A characteristic pattern of low molecular weight conjugates is obtained, which includes the ubiquitinated histone, uH2A, and unknown molecular species with M_rs of 14, 23, 26 (two bands) and 29 KD. A broad spectrum of higher molecular weight conjugates is also produced. The formation of all conjugates is absolutely dependent on ATP and, upon depletion of ATP, they are rapidly broken down. The 14, 23 and 29 KD species are found in all subcellular fractions examined. uH2A is located exclusively in the nuclear fraction. The pair of 26 KD bands is specifically associated with the ribosome fraction. A considerable percentage of the higher molecular weight conjugates sediments with the small particle (100,000 x g) fraction in the ultracentrifuge but is solubilized with deoxycholate, indicating that there are many membrane-associated conjugates. The pattern of ubiquitin conjugates revealed by immunoblotting is somewhat different from that obtained with permeabilized cells but a number of bands observed by the two methods coincide.

We have studied the relationship between ubiquitin conjugation to cellular proteins, intracellular protein degradation and the heat shock response. Upon exposure of hepatoma cells to a non-lethal heat shock at 43°C, there is a transient increase of high molecular weight ubiquitin conjugates during the first hour, which is reversed after 2-3 hours' further incubation at the same temperature. A transient fall in intracellular ubiquitin and uH2A levels coincides with the increase in high molecular weight conjugates. The transfer of the cells to 43°C is also accompanied by a brief burst of accelerated intracellular proteolysis. Possible mechanistic interrelationships between the above phenomena and the synthesis of heat shock proteins will be discussed.

343 FREE RADICALS AND PROTEIN DEGRADATION.

Roger T. Dean, Simon P. Wolff and Wendy Jessup. Cell Biology Research Group, Brunel University, Uxbridge, Middlesex, UB8 3PH, U.K.

Selective fragmentation of proteins in solution during attack of certain free radicals, notably the hydroxyl radical, will be described. Structural changes in the proteins include residue modification, and unfolding as reflected in fluorescence changes. This unfolding is also usually reflected in increased susceptibility of the protein to enzymatic hydrolysis. However, when other modifications (such as glycosylation) are concurrent with radical damage, this effect may be submerged in an increased resistance to hydrolysis. Crosslinking studies will also be described. Some data indicate that radical fluxes, by increasing the susceptibility of proteins to enzymatic degradation, may enhance overall proteolysis, but it has proved very difficult to demonstrate this in intact cells, and some such studies will be reviewed.

The situation with regard to proteins present within or adjacent to a lipid environment is very different from that of proteins in solution, such as were outlined above. Studies on selective damage to the apoprotein B present in low density lipoprotein particles, and indications of the possible functional significance of this in relation to LDL degradation and to atherosclerosis will be described. In addition, studies on the degradation of monoamine oxidase in situ in mitochondrial membranes will also be outlined. These indicate that because of the presence of lipids, attack by externally-generated free radicals is rather less effective than it is on proteins in solution, and the attack has several distinct reaction components. Thus for instance, lipid radicals themselves seem to involved in the attack on proteins in these circumstances, as are other products of radical initiated lipid peroxidation. The possible roles of antioxidants in restricting protein damage in membranes, and of other defences in restricting radical damage to proteins in solution, will be described.

344 LIPOXYGENASE AND ATP-DEPENDENT PROTEOLYSIS IN THE BREAKDOWN OF MITOCHONDRIA.
Samuel Rapoport. Institute of Biochemistry, Humboldt University Berlin, DDR-1040 Berlin, Hessische Str. 3 - 4, GDR.

Mitochondria disappear in reticulocytes by lipoxygenase (LOX) triggered ATP- and ubiquitin-dependent proteolysis.

Cell precursors in the bone marrow and highly immature reticulocytes contain the LOX mRNA in a masked translationally inactive state in form of mRNP's. Unmasking of the mRNA, presumably by a limited proteolysis of inhibitory proteins, results in the massive synthesis of the erythroid cell specific LOX.

LOX from rabbit reticulocytes has been purified and its properties characterized. Like other enzymes of this class it is a monomer of about 70 kD molecular mass and contains one non-heme Fe. The enzyme is a 15(n-6)-LOX, with a subordinate 12(n-9)-activity. Reticulocyte LOX is characterized by its capacity to attack not only free polyenoic fatty acids but also phospholipids and even intact mitochondria. In this property it differs from the other lipoxygenases of animal origin. Reticulocyte LOX is subject to auto-inactivation by its products, hydroperoxypolyenoic acids, caused by the oxidation of one essential methionine to its sulfoxide.

Immunological tests as well as hybridization experiments with a cDNA probe show the reticulocyte LOX to be specific for erythroid cell lines. Erythroid cell specific LOX mRNA's were demonstrated in bone marrow and/or red cells of mice, rats, rabbits and men. These data indicate both a common mechanism of maturation of red cells in all species which is conserved in evolution and an evolutionary divergence from lipoxygenases occurring in other types of cells.

Erythroid cell-specific LOX attacks the mitochondrial membranes and causes lysis, selective inhibitions of the respiratory chain and changes of their passive electrical properties.

The activity of LOX accounts for about 5 % of the total O_2 uptake of intact reticulocytes.

Recently it was found that the mitochondria of immature reticulocytes are non-susceptible to the attack by LOX. They require the action of a protein, the "mitochondria-susceptibility-factor", the synthesis of which during in vitro maturation depends on the supply of iron in the incubation medium.

The attack by LOX triggers the action of the ATP- and ubiquitin-dependent proteolytic system, which accounts for more than 90 % of the cellular proteolysis of intact reticulocytes. The proteolytic system attacks practically exclusively the mitochondria. Native mitochondria are a far better substrate than heat denatured ones. Proteolysis goes hand in hand with the disappearance of activities of respiratory enzymes.

345 MOLECULAR BIOLOGY OF CYSTEINE PROTEASES: PREDICTIONS FOR INTRACELLULAR CATABOLISM.
G. Salvesen (1), N. Gay (2), (1) Strangeways Laboratory, Cambridge, U.K. (2) MRC Laboratory of Molecular Biology, Cambridge, U.K.

Intracellular catabolism of proteins is thought to be accomplished by a battery of enzymes contained within lysosomes, including the well characterized cysteine proteinases cathepsin B, cathepsin H and cathepsin L. These enzymes are probably involved in the latter steps of protein degradation and may be thought of as analogues of the powerful digestive enzymes of the pancreas. Using recombinant DNA techniques we have identified a protein homologous with these enzymes and sequenced the gene encoding it. The gene exists as a single copy in both the cog and human genome, and the putative protein encoded by it contains 300-400 amino acid residues, similar in size to the pre-lysosomal forms of cathepsins B and L. RNA hybridization studies of various tissues show the distribution expected for a lysosomal enzyme and this finding is considered in terms of the, as yet unknown, activity of the protease.

346 DEGRADATION OF VERY-SHORT-LIVED CYTOSOL PROTEINS AFTER MICROINJECTION INTO CULTURED HEPATOCYTES.

Peter Bohley,Gabriele Adam and Dieter Mecke
Physiol.Chem.Inst..University D 74 TÜBINGEN Federal Republic of Germany

More than 20% of newly synthesized proteins are degraded in hepatocytes within the first hour after their synthesis. Cellular compartments as well as molecular mechanisms responsible for this rapid proteolysis are still unknown. Rat hepatocytes cultured in Williams-E-Medium were double-labelled with C-14-leucine for 15 hours. chased for 24 hours. and with H-3-leucine for 30 min..washed extensively with 0.3m sucrose and homogenized.Cytosol proteins were rapidly isolated by microscale cell fractionation by using an AIRFUGE. Autoproteolysis of these double-labelled cytosol proteins is very low after isolation (<0.4%/h for H-3-labelled proteins and 0.07%/h for C-14-labelled proteins). These substrate proteins were introduced into unlabelled cultured hepatocytes according to OKADA and RECHSTEINER(CELL 29,33-41) by using 0.5m sucrose and 10%(v/v)polyethyleneglykol 1000 in Williams-E-medium for 16 min. at 37°C followed by 3 min.incubation in hypotonic Williams-E-medium. Repetition of this hypotonic treatment markedly facilitates the selective lysis of hypertonic pinosomes and does not increase the activities of lysosomal enzymes in cytosol. All these treatments do not change the survival rates of the hepatocytes in monolayer between the 3rd and the 8th day in culture. More than 70% of the introduced proteins are found in the cytosol fraction immediately after the lysis step. The subsequent distribution and degradation of these proteins was investigated by the microscale cell fractionation and determination of protein-bound and free radioactivity in the subcellular fractions. Surprisingly,LYSOSOMES have the highest selectivity for the uptake and degradation of very-short-lived cytosol proteins.

347 CROSS INHIBITION OF ACID HYDROLASES: EFFECTS OF $[H^+]$ ON INHIBITION OF LYSOSOMAL AND NON LYSOSOMAL ARYLSULPHATASES BY PHOSPHORIC ESTERS.

Lorenzo Bolognani,Eugenio Davolio,Aurora Pederzoli and Nicola Volpi-Department of Animal Biology-Univ. of Modena-via Berengario 14-41100-MODENA -Italy.

Arylsulphatases (AS)are enzymatic glycoproteins (E.C.3.1.6.1)widely distributed in secretory and non secretory cells of vertebrates and invertebrates.Sialic acid is present in the glycoconjugate portion of the enzymes in vertebrates,whilst muramic acid was demonstrated in AS from Mollusca (Bolognani et al Comparative Bioch.Physiol.(1984)77B,89 and Sanguini et al.Comp. Bioch. Physiol.(1984)78B,533).Enzymes belonging to AS type A are present in lysosomes,but also in "tubular systems containing enzymes",as described for acid phosphatase in secretory cells(Bacsy et al. Tissue colture and RES;P.Rölich and Bacsy E. ed .(1984)103-108)and as secreted glycoconjugate enzymes.Fluctuation in AS type A and B has been described in secretory cells of the rabbit endometrium depending on sexual activity(Vitaioli et al.:Histochemistry (1985)83,127).Regulation of AS depends on translational and post-translational events.AS activity is inhibited by phospho-esters and related derivatives (phospholipids,nucleotides, etc.).The inhibition has been studied on purified enzymes and on purified lysosomal preparation (Sawant P.L.et al. B.B.A.(1964)85,82).Reciprocal inhibitory effects were observed on AS in the presence of phosphoric esters and vice versa acid phosphatases were inhibited in the presence of sulphuric esters(Bolognani et al.Biochem. International (1982)4,243). Proton concentration might be controlled by this system since the products of these enzymes are very strong acids (sulfuric and phosphoric).$[H^+]$ is also important in affecting the reciprocal inhibition:the inhibitory effect by phosphoesters on AS increases at pH 5-4 ; it disappears at neutral pH.The optimum pH of inhibited and non inhibited AS is shifted towards lower pH values by increasing temperature.

348 PROBLEMATIC ULTRASTRUCTURAL CYTOCHEMICAL DEMONSTRATION OF AcPase AND AMPase IN LYSOSOMES OF THE RAT KIDNEY PROXIMAL TUBULE CELLS.

Caesar E. Hulstaert(1), Dharamdajal Kalicharan(1), Machiel J. Hardonk(2). (1) Centre for Medical Electron Microscopy, University of Groningen, Oostersingel 69/2, 9713 EZ Groningen and (2) Department of Pathology, University of Groningen, The Netherlands.

The employment of cerium instead of lead as the capturing agent in phosphatase cytochemistry has lead to an excellent demonstration of many phosphatases in liver and kidney (Hulstaert et al. Histochemistry 78, 71, 1983; Hardonk et al. Acta Histochem. Suppl. 31, 253, 1985). Nevertheless, it appeared that lysosomal AcPase and AMPase activity could not be satisfactorily demonstrated in the rat kidney proximal tubule cells, as in most of these cells the reaction product was partly or almost completely present outside the organelles. Prolongation of GA fixation (15 and 30 min instead of 5 min), the employment of FA as fixative, freezing in liquid N_2 after fixation, omission of anaesthesia, a shorter incubation in the incubation medium (5 and 15 min instead of 30 min), incubation at 4°C, employment of CMP instead of β-glycerophosphate as substrate, incubation in the presence of DMSO or saccharose, the employment of lead instead of cerium as the capturing agent did not result in a better localization. Biochemical determination of AcPase activity in the rat liver and kidney lysosomal fractions and their supernatants gave the same percentage activity in the supernatants of both organs. Therefore it is concluded that fixation of the kidney may disturb the lysosomal membranes of the proximal tubule cells in such a way that enzymes leak out of the lysosomes into the cytoplasm.

349 THE USE OF ANTIBODIES AGAINST LYSOSOMAL CATHEPSINS B,D,H AND L: IDENTIFICATION OF PRESURSOR SPECIES.

Bernd Wiederanders, Heidrun Kirschke, Institute of Biochemistry, Medical Faculty, Martin-Luther-University, 4020 Halle, P.O.Box 184, GDR.

Polyclonal antibodies were raised in rabbits against cathepsins, B,D,H and L isolated from rat liver lysosomes. The monospecificity of the antisera has been demonstrated by two dimensional immunoelectrophoresis. The immunoglobulins did react with the related antigens from bovine and human as well, but only at 20 times higher concentrations of antigen. The antisera against the cysteine proteinases showed also weak cross reactivities with one another on nitrocellulose blots (anti-L with H, anti-H with B and L, anti-B with H and L), but they did not react in diffusion procedures.
These antisera were used to identify antigen related molecules in cultured fibroblasts which had been labelled by 14-C-Leu or 35-S-Met. The fibroblast lysates were precipitated by the immunoglobulins and the precipitates were processed for fluorography after SDS-PAGE. All four cathepsins show precursor molecules in the molecular weight range of 45-55 kDa. The precursors of the cathepsins B, H and L seem to be the dominant intracellular forms of the antigens, since they represent the most intensively labelled molecular species in the cells. They are, in contrast to the mature enzyme, yet visible after one week chase period. In contrast, the mature form of cathepsin D is the dominant intracellular antigenic species.

350 HUVE CELLS USED FOR ENZYME REPLACEMENT STUDIES IN FABRY DISEASE.

Lis Hasholt, Sven Asger Sørensen, Institute of Medical Genetics, Blegdamsvej 3, DK 2200 N, Copenhagen Denmark.

Fabry disease is an X-linked inborn error of glycolipid metabolism caused by a deficiency of α-galactosidase A (α-gal A). The glycosphingolipids, which accumulate in the body fluids of the hemizygotes without α-gal A, are deposited in the lysosomes of cells from different tissues; the accumulation in the vascular endothelium is suggested to be responsible for the major clinical manifestations of the disease. Endothelial cell cultures should therefore be advantageous for enzyme therapy studies on this defect.

The aim of the present study was to show if purified α-gal A can be internalized in HUVE cells by ConA-mediated endocytosis. HUVE cell lines were established from cells isolated from the cords of normal new-borns and a Fabry hemizygous fetus after abortion. The hemizygous HUVE cells express the gene defect as they are deficient in α-gal A. Binding of FITC-ConA to the HUVE cell surfaces at 4°C followed by incubation at 37°C showed that ConA redistributed in minicaps and was internalized within 30 min at 37°C. Then the cells were exposed to Con A and thereafter incubated in medium supplemented with purified α-gal A; quantitative measurements of the activity showed, that the enzyme had been taken up. An α-gal A activity corresponding to the normal level for HUVE cells was obtained in the Fabry cells by this treatment. To investigate the stability of the internalized enzyme the cells were incubated in normal culture medium after the addition of α-gal A. The supplied α-gal A activity was reduced to about 15 per cent after 24 h, but 10 per cent activity still remained after 72 h.

HUVE cells: Human umbilical vein endothelial cells.

351 DEGRADATION OF THE THYROID HORMONE PRECURSOR PROTEIN IN THYROID LYSOSOMES. EVIDENCE FOR A HIGH MOLECULAR WEIGHT PARTIALLY-HYDROLYZED THYROGLOBULIN INTERMEDIATE.

Bernard Rousset, Samia Selmi and Rachida Rabilloud
INSERM U.197, Faculté de Médecine Alexis Carrel. 69372 Lyon Cédex 08. France

Secretion of hormones by the thyroid gland requires the intralysosomal proteolysis of a high molecular weight precursor protein (a dimer of 660,000 daltons): thyroglobulin(Tg). Tg stored in the intra follicular space (colloid) is taken up by the cells by a mechanism of endocytosis ; resulting intracellular vesicles fuse with lysosomes to give secondary lysosomes or phagosomes. We recently reported (Biochem.J. 1985, 232, 529) a procedure to obtain highly purified preparations of thyroid lysosomes which contain immunoassayable Tg. In the present study, we have characterized intralysosomal Tg (TgL) in terms of structure and biological properties and compared TgL to colloid Tg (TgC) and to non-iodinated newly-synthetized Tg: microsomal Tg (TgM). Pig thyroid lysosomes were purified by isopycnic centrifugation on iso-osmotic Percoll gradient. Enrichment in acid hydrolase activities was close to 50. TgL represented 5-10 % of soluble intralysosomal protein released by osmotic shock. TgL was analyzed in terms of : a) size by PAGE and immunoblotting using polyclonal anti-pig Tg antibodies, b) sedimentation coefficient by ultracentrifugation on sucrose gradient and radioimmunoassay. Under non-reducing conditions, TgL had the same apparent molecular weight of 280,000 as TgC and TgM, but a lower S value: 17 S as compared to 18 and 19 S for TgM and TgC, respectively. The iodine content of TgL (0.25 %) was intermediate between that of TgM ($<$ 0.05 %) and TgC (0.8 %). Under reducing conditions (+ DTT), the apparent molecular weight of TgC and TgM remained close to 280,000, whereas TgL gave rise to low molecular weight species (mainly below 50,000 daltons). These data show that a) Tg exists as a high molecular component in lysosomes but this Tg (TgL) is clearly distinct from both microsomal Tg (TgM) and colloid Tg (TgC) and b) TgL is cleaved at numerous sites but the proteolytic products remain attached by disulfide bonds in a molecular species the size of which is close to that of native Tg.

352 MANNOSIDOSIS FIBROBLASTS ARE CORRECTED METABOLICALLY BY DIRECT TRANSFER OF α-D-MANNOSIDASE FROM DONOR LYMPHOCYTES OR BY ENDOCYTOSIS OF NORMAL ENZYME.

D. Abraham*#, I. Cenci di Bello*, H. Muir#, I. Olsen# and B. Winchester*
*MRC Human Genetic Diseases Research Group, Department of Biochemistry, King's College London, United Kingdom. #Kennedy Institute of Rheumatology, London, United Kingdom.

Fibroblasts from patients with mannosidosis, the lysosomal storage disease resulting from an inherited deficiency of lysosomal α-D-mannosidase (EC 3.2.1.2.4) accumulate specific mannose-containing storage products which are characteristic of the disease (1). These oligosaccharides were extensively degraded following transfer of lysosomal α-D-mannosidase from lymphocytes to mannosidosis fibroblasts by a process which required cell-to-cell contact. Prolonged correction of the metabolic abnormality of the recipient cells was sustained if contact with fresh donor lymphocytes was periodically renewed (2). The specific storage products were also dispersed after endocytosis of purified human liver acidic α-D-mannosidase B (3). The rate and pattern of breakdown of the specific mannose-rich oligosaccharides throw light on the catabolic pathway of asparagine-linked glycans. The sub-cellular location and molecular form of the normal α-D-mannosidase transferred by these two processes have been studied. These observations may be highly relevant to lymphocyte function in enzyme replacement therapy by transplantation procedures currently being attempted.

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353 IMMUNOCYTOCHEMICAL LOCALIZATION OF THE LYSOSOMAL ENZYME α-GLUCOSIDASE IN HUMAN ENTEROCYTES.

J.A.M. Fransen (1), J. Klumperman (1), L.A. Ginsel (1), R.P.J. Oude Elferink (2), J. Hilgers (3), and J.M. Tager (2). (1) Laboratory for Electron Microscopy, University of Leiden, Rijnsburgerweg 10, 2333 AA Leiden, (2) Laboratory for Biochemistry, University of Amsterdam, P.O. Box 20151, 1000 HD Amsterdam, (3) Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

In an investigation of the intracellular transport of glycoproteins in polarized epithelial cells, we have studied the localization of the lysosomal enzyme α-glucosidase in human enterocytes using electron-microscopic immunocytochemistry (1).

With a polyclonal antiserum we found labelling in the lysosomes, Golgi apparatus and, unexpectedly, in the brush border. When instead a mixture of monoclonal antibodies against α-glucosidase (2) was used, an identical labelling pattern was obtained. The individual monoclonal antibodies showed the following labelling patterns: 43G8, lysosomes only; 43D1, lysosomes and microvilli; 118D2E8, Golgi apparatus and glycocalyx but not lysosomes.

Biochemical studies show that 43G8 only reacts with the 76 KD mature form of α-glucosidase, and 43D1 reacts with all forms of α-glucosidase. We therefore conclude that a precursor-like form of the enzyme is present in the brush border.

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354 EM-IMMUNOCYTOCHEMICAL LOCALIZATION OF A BRUSH-BORDER AND LYSOSOMAL ENZYME IN TWO HUMAN COLON CARCINOMA CELL LINES.

J. Klumperman (1), J.A.M. Fransen (1), H.P. Hauri (2), R.P.J. Oude Elferink (3), J.M. Tager (3), L.A. Ginsel, (1). (1) Laboratory for Electron Microscopy, University of Leiden, Rijnsburgerweg 10, 2333 AA Leiden, The Netherlands, (2) Biocentre of the University of Basel, Switzerland, (3) Laboratory for Biochemistry, University of Amsterdam, P.O. Box 20151, 1000 HD Amsterdam, The Netherlands

Caco-2 and HT29 are cell lines derived from human colon carcinomas. Caco-2 cells show enterocytic differentiation and synthesize brush-border enzymes (1). A sub-cell line of HT29, when cultured on medium without glucose, also shows enterocytic differentiation (2). The differentiation of these cells is characterized by the presence of tight junctions and microvilli, which can be seen in electron micrographs.

Using electronmicroscopic immunocytochemistry we studied the localization of the brush-border enzyme sucrase-isomaltase and the lysosomal enzyme α-glucosidase in these cells. In both cell lines sucrase-isomaltase was visualized in the Golgi apparatus, lysosomes and microvilli.

A polyclonal antiserum to α-glucosidase showed a labeling of the Golgi apparatus, lysosomes and microvilli. α-glucosidase could also be visualized with two monoclonal antibodies. One of them (43D1) recognized all forms of the enzyme and reacted with the microvilli and the lysosomes. The other monoclonal antibody (43G8) recognizes the mature form of α-glucosidase (76KD) and labels only the lysosomes (3). These data lead to the conclusion that the labeling of the microvilli is due to a precursor form of α-glucosidase.

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355 AUTOFLUORESCENCE AND DEMONSTRATION OF ACIDITY BY ACRIDINE ORANGE STAINING IN DIFFERENT TYPES OF LARGE VACUOLES IN THE SMALL INTESTINE OF THE SUCKLING RAT AND PIG. Baintner K. Research Institute for Animal Nutrition, 2053 Herceghalom, Hungary.

Three sorts of intestinal large vacuoles were investigated, one with transport function /"eosinophilic droplet" of the newborn pig/ and two with probable digestive function /the supranuclear vacuole of the suckling rat and the "empty vacuole" of the suckling pig/; the latter vacuoles contain hydrolases with acidic pH optima.

The vacuoles in the cells from the distal small intestine of the suckling pig and especially those of the suckling rat showed yellow autofluorescence, the "eosinophilic droplets" of the newborn pig did not.

When enterocytes were vitally stained with acridine orange, the nuclei showed yellow-green and the vacuoles red or orange fluorescence. Prolonged illumination damaged the cells and terminated the vacuolar fluorescence, possibly due to the collapse of the pH gradient. Non-vacuolar red fluorescence was unaffected by prolonged illumination.

It is concluded that the large digestive vacuoles of the distal small intestine of young mammals have an acidic interior, which provides favourable environment for the function of digestive enzymes. These experiments support the notion that large vacuoles originate from lysosomes. The acidic pH in the "eosinophilic droplets" transporting IgG and other colostral proteins was unexpected.

356 RENAL DEPLETION OF THE FOOD PROTEINS ABSORBED BY INTESTINE.
X.A. Zufarov. Histology Department, Medical Institute, Tashkent-48, K.Marx Str.103, USSR.

The exogenous and food proteins are known to be absorbed undepleted by a small intestine at the early postnatal ontogenesis. These proteins penetrate the enterocytes means of pinocytosis, accumulate in the Golgi complex and in the form of isolated vesicles are extruded into the intercellular space, which serves a depo for the absorbed proteins accumulated there. Gradually, the accumulated material passes into the lymphatic capillaries. As the absorbed protein passes unchanged the whole route, it was suggested that there existed an organ or tissue where the endogenous depletion of it had to take place. For knowing it, the radiologic studies using labelled milk proteins were made. The kidney was revealed to be the main radioactive site. An electron microscopic investigations of kidneys at various intervals after the per os administration of food proteins to the newborn rats were undertaken. Appearence of many electron dense, highly acid phosphatase-positive lysosomes, containing proteins were found in the proximal tubular cells. The immune morphologic techniques demonstrated that the per os administered proteins preserved their antigenic structure in the kidneys. Biochemical analysis of the kidney homogenates indicated to sharp elevation of their proteolytic activity after both the per os and parenteral introduction of proteins. The proteolytic activity, however, was higher after the per os administration. This might be the result of absorbtion of both food proteins and pancreatic digestive enzymes in intestine and their activization in the renal lysosomes. The obtained data demonstrated that in early postnatal ontogenesis the kidneys were responsible for the food proteins hidrolysis.

357 X LYSOSOMAL PROTEINASE ACTIVITIES IN EMBRYONIC SKELETAL MUSCLE.
I.Sohar(1), Eva Fekete(1), J.W.C. Bird(1), Glee Yorke(2), F.J.Roisen(2). Bureau of Biological Research, Rutgers State University(1) and Department of Anatomy, UMDNJ-Rutgers Medical School,(2), Piscataway, NJ 08854, USA

Previous studies have demonstrated significant increase in cysteine proteinase activities during the fusion of cultured myoblasts to form myotubes. The cultures were either from 11 day-old dissociated embryonic breast muscle or the established myogenic cell lines L6 and L8.

In the present study, cathepsin B and L activities were measured in embryonic chicken breast muscle tissue from 10th to the 20th day of embryonic life. Total enzyme activity increased until the 18th day, and dropped before hatching at the 20th day. Specific enzyme activity increased until the 14th day of incubation and then decreased progressively until hatching of the egg. The results suggest a role for the cysteine proteinases during the differentiation of embryonic skeletal muscle, and are similar to those reported for muscle cells differentiating in culture conditions. The accelerated protein synthesis and endogenous cysteine proteinase inhibitors remains to be demonstrated.

Present address: Department of Biochemistry, Medical School Szeged,Hungary

358 LYSOSOMES IN ATROPHYING AND HYPERTROPHYING SKELETAL MUSCLE

Anna Jakubiec-Puka, Jolanta Kordowska
Laboratory of Protein Metabolism, Nencki Institute of Experimental Biology, 02-093 Warszawa, ul. Pasteura 3, Poland

Ultrastructure was studied in the rat skeletal muscles undergoing several different cathabolic and anabolic processes, i.e. in muscle atrophying following denervation, tenotomy or maintenance in shortened position, as well as in muscle hypertrophying following maintenance in extension or in muscle recovering from denervation atrophy after reinnervation. An increased amount of lysosomes was observed in all the mentioned muscles as compared with controls. In parallel with observations of lysosomes, changes in ultrastructure of the contractile apparatus were studied and the activity of proteolytic enzymes was measured in these muscles The particular results were compared and discussed to elucidate the possible ways of degradation of muscle proteins and substructures of the contractile apparatus.

359 APOPTOSIS IN MUCOID EPITHELIUM OF THE MOUSE

N. Pipan, M. Sterle. Institute of Human Biology, Medical Faculty, Lipičeva 2, 61000 Ljubljana, Yugoslavia

In the gastric epithelium of 14–18 day old embryos and 2–6 day old mouse the formation of apoptotic cells and their phagocytosis by viable sister cells was analysed. For the observation of topographic relations between apoptotic and normal epithelial cells with SEM, the tissue was cracked before coating with gold. In the analysis with TEM cytochemical methods for identifications of surface carbohydrates and different tracers for apical and lateral cell membranes were applied. Apoptotic cells were found on apical, lateral and basal surfaces of the epithelium. Ruthenium red stained strongly all accessible surfaces of normal cells and of apoptotic bodies. The quantity of neutral mucosubstances, as revealed by staining with tannic acid-uranyl acetate seems to diminish in the glycocalyx of apoptotic cells. Results achieved with substances that stain carbohydrates or label apical and lateral membrane as also the analysis of cracked tissue with SEM suggest, that the phagocytic vacuoles arise on the lateral cell side. The phagocytic activity is not dependent or restricted to a definite age or differentiation step of mucoid cells.

360 SUBCELLULAR FRACTIONNATION OF MORRIS HEPATOMA 7777 CELLS.

F.Mainferme and R.Wattiaux. (Facultés Universitaires, Namur, Belgium).

The large number of early intermediates formed during the biosynthesis of lysosomal cathepsin C are found to be mainly distributed in two fractions, one containing the ER and Golgi apparatus while the other is a lysosome enriched fraction. (Mainferme et al. Eur.J.Biochem. 153, 211-216, 1985). In order to establish a more precise subcellular distribution of the cathepsin C precursor polypeptides a systematic study of the subcellular fractions of in vitro cultivated Morris Hepatoma 7777 cells was started by us.

The distribution of the marker enzymes for mitochondria, lysosomes, peroxisomes and microsomes seem to indicate that homogenates of hepatoma 7777 cells can be fractionated by differential centrifugation in a manner similar to that used for normal liver cells. However one of the major differences that exists between the normal and hepatoma cells is that in the case of the latter there is a lower percentage of proteins in the heavy mitochondrial fraction resulting in a high relative specific activity for cytochrome oxidase, on the other hand there is a comparatively higher percentage of proteins in the light mitochondrial fraction leading to a lowering of the relative specific activity for cathepsin C.

After isopycnic centrifugation of the postnuclear supernatants of hepatoma 7777 cells in sucrose gradients, galactosyltransferase equilibrates at a mean density of 1.128 g/ml, cytochrome oxidase forms a single peak at a density of 1.174 g/ml, cathepsin C is recovered as a band centered around a density of 1.174 g/ml and NADPH cytochrome C reductase bands in a density region ranging from 1.174 g/ml to 1.204 g/ml. Thus the mitochondria and lysosomes of the hepatoma 7777 cells are somewhat lighter than those present in normal liver cells. However in Nycodenz gradients cathepsin C is recovered in lower density regions (1.09 g/ml) which is the same position as galactosyltransferase while NADPH cytochrome C reductase is found to equilibrate at a density of 1.169 g/ml.

361 LYSOSOMAL LOCALISATION AND ESTROGEN CONTROL OF THE SYNTHESIS AND PROCESSING OF A 52-kDa GLYCOPROTEIN SECRETED BY HUMAN MAMMARY CANCER CELLS. M. Morisset, F. Capony, F. Vignon, M. Chambon, H. Rochefort. Unité d'Endocrinologie Cellulaire et Moléculaire (U 148) Institut National de la Santé et de la Recherche Médicale, 60 rue de Navacelles, 34100 Montpellier France.

A 52-kDa glycoprotein is secreted by human breast cancer cells in culture after estrogen stimulation (Westley and Rochefort, Cell 20:352, 1980). Using monoclonal antibodies, we have quantified and characterized the corresponding proteins of the cell compartment. Using pulse chase experiments, we show that the 52-kDa protein is secreted for 30 % and the majority is processed into a 48-kDa and 34-kDa protein. The processing is inhibited by lysosomotropic agents and leupeptin suggesting that it is occuring in acidic vesicles like lysosomes or endosomes. The presence of mannose-6-phosphate signals on the 52-kDa and morphological studies indicate a lysosomal localization.

Estradiol increased the intracellular level of immunoreactive 52-kDa related proteins by 4-fold. The stimulatory effect on |^{3}H|mannose incorporation was similar and the endo H sensitivity of the proteins, was not altered, suggesting that estrogens did not modulate the glycosylation step. Antiestrogens were inactive. Estradiol also increased the stability of the 52-kDa precursor as well as that of total proteins.

We conclude that the secreted 52-kDa protein is a precursor of two cellular proteins, its production is stimulated by estradiol that increases its synthesis and decreases its processing.

362 SUBCELLULAR COMPARTMENTATION OF DOLICHOL UPTAKEN BY MOUSE LEUKEMIA CELLS Anna Szkopińska, Wiesław Jankowski, Ewa Swieżewska. Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 02-532 Warszawa, Rakowiecka 36, Poland

Tritiated dolichol was intraperitoneally coincubated with mouse leukemia Lcb 35 cells. Preliminary sucrose gradient centrifugation separated uptaken radioactivity into two fractions: cytoplasmic and membrane-bound one. Further combined centrifugations on sucrose and metrizamide gradients allowed to purify membraneous fraction Enzymatic analysis showed it to be lysosomes. Strong support for that conclusion were the results obtained from experiments in which density of lysosomes was selectively changed by pretreatment the animals with Triton WR 1339. In that case radioactivity migrated to the lower density part of sucrose gradient. Thin layer chromatography of radioactive lipids present in membraneous fraction revealed that dolichol was mostly unchanged and partly esterified with fatty acids.

363 OVINE CEROID-LIPOFUSCINOSIS : AN INHERITED DEFECT IN LYSOSOMAL PROTEOLYSIS? D.N. Palmer, D.R. Husbands, G. Barns, R.D. Jolly. Department of Veterinary Pathology and Public Health, Massey University, Palmerston North, New Zealand

The ceroid-lipofuscinoses are inherited lysosomal storage diseases of children and animals characterised by a fluorescent lipopigment stored in a variety of tissues. Defects in lipid metabolism or the control of lipid peroxidation have been postulated to explain their pathogenesis. In the present study lipopigment was isolated from liver, kidney, pancreas and brain of sheep affected with ceroid-lipofuscinosis.

The lipopigment phospholipids contained the same species as normal lysosomes and included bis(monoacylglycero)phosphate, a lysosomal marker. Similarly the neutral lipids, notably dolichol, ubiquinone and dolichyl esters were typical of those in lysosomal membranes. The remaining material, accounting for approximately two thirds of the lipopigment mass, was proteinaceous. Sodium dodecyl sulphate polyacrylamide gel electrophoresis showed a major band of M_r 14,800, heterogenous material between 5,000 - 9,000 M_r and a major band of M_r <3,500. The presence of the 3,500 M_r proteins in whole affected tissue homogenates distinguished them from homogenates of normal tissues. It was concluded that low M_r proteins are specifically stored in ovine ceroid-lipofuscinosis and that the ceroid-lipofuscinoses may result from defects in protein catabolism or its control.

364 IMPORTANCE OF LYSOSOMES IN THE DEVELOPMENT OF RADIATION-INDUCED DAMAGE OF NEURONS.

Vojtěch Kamarád (1), Václav Svoboda (2). (1) Department of Histology, Medical Faculty, Palacký University, 775 15 Olomouc, and (2) Purkynje Medical Research Institute, 500 00 Hradec Králové, Czechoslovakia.

The paper deals with the importance of lysosomes during development of post-radiation changes in neurons of rat and mice cerebral cortex at early stages after irradiation with high supralethal doses.

The experimental animals were exposed to a local head- and whole-body irradiation receiving a dose of 50 to 960 Gy from gamma emmitor Co^{60}. The samples from cerebral cortex were withdrawn immediately till six hrs after irradiation. Routine electron microscopic examination and histochemical determination of some lysosomal hydrolases were performed.

An increased activity of acid phosphatase was proved histochemically in lysosomes and occured also diffusely in neuron cytoplasm. Reaction of nerve cells was of mosaic character. Besides neurons with high activity of acid phosphatase there were cells displaying the activity similar to that of control animals. The electron microscopic examination showed a corresponding multiplication of lysosomes in perikarya of neurons after irradiation.

Activation of lysosomal apparatus of neurons is not considered to be a primary effect of radiation. According to our opinion it is due to metabolic changes in the cerebral cortex induced by changed permeability of hematoencephalic barrier.

365 LYSOSOMAL CHANGES RELATED TO 20-HYDROXYECDYSONE AND JUVENILE HORMONE TREATMENTS DURING THE METAMORPHOSIS IN THE MIDGUT OF *GALLERIA MELLONELLA* /INSECTA, LEPIDOPTERA/.

László G. Kőmüves, Miklós Sass, János Kovács. Department of General Zoology, ELTE University, H-1088 Budapest, Puskin u. 3., Hungary.

Programmed cell death of larval organs during metamorphosis of insects is accompanied by changes of lysosomal activity. We have studied the developmental changes in activity of lysosomal enzymes and their hormonal regulation in the midgut of *Galleria mellonella*.

Total, as well as specific activities of various acid hydrolases /acid phosphatase, cathepsin D, acid β-galactosidase, acid ribonuclease/ increased gradually during the last instar reaching the maximum on the 7th day. Administration of an analogue of *juvenile hormone* /JHA/ on the 3rd day supressed this increase. *20-hydroxyecdysone* /20-HE/ treatment for 3 h to 5 day old larvae slightly elevated the activity of enzymes both in the controls and in the JHA pretreated larvae. The proportion of free, osmotically releasable and unreleasable activities of acid hydrolases did not change until the 6th day of the last larval stage, when the free and releasable activity strongly increased. Administration of JHA on the 3rd day prevented this process. 20-HE treatment on day 5 led to the redistribution of enzymes in favour of the free and releasable fractions in the control as well as in JHA treated larvae.

These observations corroborate the conclusion that 20-HE regulated redistribution of lysosomal enzymes is an important process in the initiation of the degradation of larval organs during the metamorphosis in insects.

366 20-HYDROXYECDYSONE INDUCED PHOSPHORYLATION OF PROTEINS IN INSECT FAT BODY CELLS

G.Csikós, M.Sass, L.Kőmüves, J.Kovács. Dept. of Gen. Zoology, ELTE Univ., H-1088 Budapest, Puskin u.3., Hungary

20-hydroxyecdysone (20-OHE) administered to 3 days old larvae of Mamestra brassicae induced autophagocytosis in the fat body cells and a rise in their cAMP content and enhanced the activity of adenylate cyclase in this tissue. When fat bodies of control and 20-OHE treated animals were homogenized and incubated in the presence of P32-ATP selective phosphorylation of a band containing proteins with the apparent m.w. of 56 000 was observed on the electrophoretogram prepared from the postnuclear fraction of hormone treated animals by SDS-PAGE method.

Actinomycin D applied 3, 6, 12 hrs before the injection of 20-OHE did not inhibit the induced autophagy and the selective phosphorylation of the above mentioned protein(s). However, it had a preventive effect on both processes when administered 21 hrs prior to the hormone. It seems probable that the rise of cAMP content and the selective phosphorylation induced by 20-OHE has a significant role in the regulation of autophagocytosis in larval tissues of insects and actinomycin D treatments point to the involvement of RNA-dependent processes in these events.

367 EFFECT OF MONENSIN ON MONOLAYERS OF RAT HEPATOCYTES.

Jacqueline Thirion and R. Wattiaux. (Facultés Universitaires, Namur, BELGIUM).

The addition of monensin (1 to 25 µmol/l) to the culture medium of one day hepatocyte monolayers induces a progressive vacuolation of the cells. This phenomenon is both time and dose dependant. Owing to the ionophoric properties of monensin, these vacuoles could originate from Golgi, endosomal or lysosomal structures.

After a 24 h. cultivation in the presence of the drug several enzymatic activities are modified, the extent of this modification is also related to the dose used, galactosyltransferase and lysosomal hydrolases activities (β-galactosidase, cathepsin C, acid phosphatase) decrease strongly. Their subcellular distributions are slightly modified. Golgi partially shifts from the microsomal fraction to the mitochondrial and nuclear fractions. Lysosomes become fragile, as a consequence, higher levels of enzymatic activities are recovered in the soluble fraction.

The sedimentation properties of the lysosomes of cells cultivated 24 h in the presence of monensin (10 µmol/l) were analysed. After isopycnic centrifugation in sucrose gradients their equilibrium density was lower (1.19 g/cm^3) than in the control cells (1.21 g/cm^3). This data was confirmed by the change of α, β, and ρ_d factors of the particles as calculated from the analysis of these lysosomes in glycogen gradients with varying concentrations of sucrose solutions as solvents. (Beaufay,H. and Berthet,J. (1963) Biochem.Soc.Symp. 23, 66-85).

However, after 24 h. of ^{14}C sucrose incorporation in the presence of the drug, the radioactivity was associated with these modified lysosomes. Under these experimental conditions, endosome-lysosome traffic does not seem to be inhibited, contrary to the effects of low temperature (17°C) which reduces sucrose incorporation and maintains it in a prelysosomal compartment.

368 LYSOSOMOTROPIC AGENTS AS REGULATORS OF LYSOSOMAL HYDROLASE ACTIVITIES.

Anatole Bulychev, Olga Assinovskaya, and Elisabeth Semenova. Institute of Cytology, Academy of Sciences of the USSR, 194064 Leningrad, 4 Tikhoretsky pr., USSR

A study was made of the influence of Nautral Red (NR), Acridine Orange (AO), chloroquine (Ch) and daunorubicin (DNR), accumulated in lysosomes of fibroblasts L, on the activity of cathepsins B and D (cB and cD), acid phosphatase (AP), N-acetyl--ß-glucosaminidase (Naßgase), and acid lipase (AL). Enzymatic activity was determined in the medium with Triton X-100 at 37°C and at the optimal pH values. The incubation of cells with NR ($1.0 \cdot 10^{-4}$ M, for 6 h) caused 34, 45, 40, 56 and 34 per cent inhibition of cB, cD, AP, Naßgase, and AL activities, resp. Corresponding results obtained for other three compounds were as follows: for AO ($5.4 \cdot 10^{-5}$ M, for 3h) 71, 49, 37, 14, and 40; for Ch. ($5.0 \cdot 10^{-5}$ M, for 3 h) - 40, 33, 17, 22, and 30, for DNR ($3.5 \cdot 10^{-6}$ M, for 3 h) - 12, 29, 27, and 21%, and no inhibition. Of special interest is our finding of unexpected stimulation of AL activity by 58 per cent in the latter case, which needs further investigation. By changing the lysosomotropic agent concentration or the incubation time, it seems possible to control lysosomal enzyme activities. The data obtained make it possible to consider these substances as possible regulators of functional activity of lysosomal machinery of the cell.

369 ANTIBODIES TO THE VACUOLE MEMBRANE.

Danièle Marty-Mazars, Christiane Périkian, Hubert Reggio and Francis Marty. Laboratoire de Biologie de la Différenciation Cellulaire - Faculté des Sciences de Luminy - 13288 Marseille Cedex 9 - France.

Rabbits were immunized with membrane fractions from the vacuole (VM) of cells from either beetroot (Beta vulgaris L. var. esculenta) or cauliflower (Brassica oleracea var. botrytis) by injection into the popliteal lymph nodes. The antisera were then tested by indirect immunofluorescence on frozen sections of tissue. Antibodies to cell components other than the vacuole system were removed by suitable absorption steps. These steps were carried out until the pattern of fluorescent labeling was that expected for the vacuole. These antibodies were directed against a small number of membrane polypeptides. When frozen sections of cells from Brassica oleracea were labeled with anti-VM antibodies followed by sheep anti-rabbit IgG conjugated to rhodamine, the fluorescence pattern lined up the vacuole periphery, but the cell cytoplasm and the nucleus were free of label. The anti-VM antibodies were directed against six polypeptides with apparent molecular weights of 26,000, 43,000, 46,000, 64,000, 80,000 , and 110,000. The vacuole system in differentiated cells from the shoot apex of Beta vulgaris comprises a large central vacuole which occupies most (>90 %) of the cell volume. The anti-VM antibodies labeled the vacuole boundary alone at the light microscope level. They were directed against two polypeptides with apparent molecular weights of 23,000 , and 26,000. We have screened for antibodies that recognized common antigens in the vacuole membrane and not those specific for a particular tissue or species. Examples are presented showing that the antibodies raised against the vacuole membrane of cauliflower recognized two VM polypeptides from beetroot with apparent molecular weights of 80,000 , and 110,000. Antibodies raised against the cauliflower VM were affinity-purified by using beetroot VM immobilized on HMD Ultrogel.

370 THE LOCALIZATION OF "LYSOSOMAL" ENZYMES AND THE PROCESS OF ROOT DIFFERENTIATION. K. Beneš. Institute of Experimental Botany, Czechoslovak Academy of Science, Ke dvoru 15, 166 30 Praha 6, Czechoslovakia.

The relation was studied between the localization of hydrolytic enzymes and the process of root differentiation. Acid phosphatase, carboxyl esterase, aryl sulphatase and various glycosidases were revealed in free floating frozen sections of Ca formol fixed root tips of Vicia faba, Zea mays and other plants, using in situ colour reactions, mainly simultaneous azocoupling. In some cases, distinct differences were observed in the activity of particular parts of the meristem. Examples were also discerned when the enzyme was found to be present in one but absent from another part of the section. The localization corresponded here to the differentiation within the histogen. Concerning the regulatory mechanisms involved in the establishment of enzyme localization patterns, the presumption of a single switch (like a formative mitosis) does not correspond to our recent results (the disappearance of aryl sulphatase activity along a particular row of cells in longitudinal section of maize root).

Using electrophoresis in polyacrylamide gels attempts were made to characterize the processes of cell growth and maturation. Though distinct differences in isoenzyme patterns between meristematic and elongation and maturation zones were revealed in some cases, no general conclusion was reached concerning the number and position of isoenzyme bands comparing the mentioned parts of the root.

371 LIPIDPEROXIDATIVE AND PROTEOLYTIC PROCESSES IN FUNGI UNDER THE INFLUENCE OF XENOBIOTICA

Hanna M. Müller, W. Edlich, H. Lyr. Institute of Plant Protection Research, Academy of Agricultural Sciences of the GDR, GDR - 1532 Kleinmachnow, Stahnsdorfer Damm 81

As a model system for investigation of peroxidativ effects in the phytopathogenic fungi Botrytis cinerea in submers culture was used. Investigated was the influence of hydrogenperoxid with biochemical and cytological methods.

The hyphae showed significant changes of the ultrastructure: swelling and vacuolization of mitochondria, loss of their ribosomes, vesiculation of endoplasmic reticulum, degradation and lysis of the cell ribosomes and deposition of lipid droplets on the membranes and in the cytoplasme dependent on concentration of the peroxid. Same effects with higher injurie of the membrane system are found in Phytophthora infestans, parallel investigation on fibroblasts showed similar effects: pathological changes of mitochondria, but also pycnosis in the nucleus.

Comparative ultrastructural investigation were performed in cultures of Phythophthora infestans with tetrachlorcarbon and chloroneb, a specific fungicide substance with a new mode of action. Scavengers like α-tocopherol and piperonylbutoxid inhibit the toxic effect of the investigated substances in various degrees.

Results are discussed in regard to the effect of a lipidperoxidative and proteolytic attack in pesticides of unknown mechanism of action.

372 A PRIMARY STUDY ON THE ISOENZYMES OF HORESHOE CRAB (Tachypleus tridentatus Leach). ASCIDIA (Styela sp.) AND AMPHIOXUS (Branchiostoma belchery Gray).

Wang Fen, Chen Yuan-Lin, Wang Deyao (Quang Teyio). Institute of Cell Biology, Xiamen University, Xiamen, Fujian, P.R.C.

Although horeshoe crab, ascidia and amphioxus are very important in their evolutionary position, the isoenzymes as a directly products of gene are rarely studied. In order to understand some common zymogrames approved to be a very useful genetic marker, and the speciality of the zymogrames of the animals for further studying, the zymograme of esterase, acid phosphotase, malat dehydrogenase, and latate dehydrogenase of horeshoe crab, ascidia and amphioxus were studied by using vertical polyacrylamide thinner layer gel electrophoresis. The results are as follows: 1). There were species specificity and tissue specificity of the isoenzyme pattern of the animals, and the zymogrames also showed differences among different sexes and ages of these animals; 2). The esterase isoenzyme pattern of amphioxus was more similar to that of ascidia than of horeshoe crab, but the others (ACP, MDH, LDH) were not; 3). The results didn't support the polyploidisation hypothesis of Ohno. We have made some discussions about the results.

373 TUBULAR LYSOSOMES AND LYSOSOMOPHAGY IN MACROPHAGES INDUCED BY MICROTUBULE DISRUPTING DRUGS AND GLUCOCORTICOIDS

Vladimir Bumbaširević, Vesna Lačković, Slobodanka Milićević, Milorad Japundžić
Institute of Histology, Medical Faculty, Višegradska 26, 11000 Belgrade, Yugoslavia

Ultrastructural and cytochemical analysis of splenic and thymic macrophages of Wistar rats, treated by 0.5 mg of colchicine, 5 mg of vinblastine-sulphate or 4 mg of dexamethasone per 100 g B.W. was performed. Marked activation of both splenic "tingible body" macrophages and thymic cortical macrophages, containing numerous phagocytosed apoptotic lymphocytes, was found 3-6 h after either drug administration. Microtubule disrupting drugs did not interfere with lysosome-phagosome fusion and degradation of phagocytosed material, as shown by AcPase cytochemistry. In later stages of degradation (12-24 h) numerous tubular lysosomes with specific morphological features, occasionally linked with larger secondary lysosomes, were seen to segregate large parts of electron lucent cytoplasm, often containing small lysosomes (lysosomophagy). As lysosomophagy was found using both types of drugs, we believe that it represents a special kind of cellular mechanism involved in regulation of cellular volume and number of lysosomes in macrophages, driving larger activated macrophages to steady state.

374 INHIBITION OF HEPATIC PROTEIN DEGRADATION BY ACUTE AND CHRONIC ETHANOL TREATMENT.

A. R. Pösö (1) and G. E. Mortimore (2). (1) Department of Biochemistry, College of Veterinary Medicine, POB 6, SF-00551 Helsinki, Finland and (2) Department of Physiology, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pennsylvania 17033, U. S. A.

Accumulation of protein into the liver after chronic ethanol administration is well established, but the mechanism is not clear. We studied the effect of ethanol on hepatic proteolysis, because according to recent evidence protein degradation, rather than synthesis, is the primary site for the regulation of cytoplasmic growth. Acute effect of ethanol on protein degradation was measured in rat livers perfused in the single-pass mode. Ethanol inhibited, in a concentration independent manner, both amino acid deprivation-induced proteolysis as well as basal proteolysis in the presence of ten times normal plasma concentration of 20 amino acids. The latter can be taken as an indication of direct inhibitory effect of ethanol on the lysosomal function. Electron microscopic examination of the lysosomal components indicates that there is more than one inhibitory site for ethanol. In chronic experiments rats were fed ethanol for 3 or 10 weeks and the rate of proteolysis was measured in 15 min cyclic perfusions in the presence of cycloheximide. Both treatments increased the amount of hepatic protein. In ethanol fed rats the rate of proteolysis was inhibited by 30 %, but only when ethanol was present in the blood of rats at the time of perfusion. Taken together, these results show that ethanol inhibits protein degradation in rat liver and this inhibition may play a significant role in the hepatic protein accumulation caused by chronic ethanol treatment.

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375 INTRACELLULAR FATES OF MICROINJECTED PRECURSOR AND MATURE POLYPEPTIDES

M. J. Gaskell, R. J. Mayer. Department of Biochemistry, University of Nottingham, Nottingham NG7 2UH, U.K.

Erythrocyte-mediated microinjection has been used to introduce several proteins into 3T3-L1 cells. Microinjected in vitro translated precursor polypeptides distribute in digitonin-extractable (cytosol), Triton X-100-extractable (organelles, vesicles and membranes), potassium iodide-extractable (actin and associated proteins) and inextractable residue (intermediate filaments/nuclear skeleton) cell fractions in growing 3T3-L1 cells. Little or no degradation is observed over a 70 hour time course. In confluent cells microinjected polypeptides distribute predominantly in the digitonin-extractable and inextractable residue fractions, but again are not degraded. In contrast, [^{125}I]-bovine serum albumin and reductively methylated [^{3}H]-haemoglobin microinjected into 3T3-L1 cells distribute predominantly in the digitonin-extractable fraction and are degraded by mechanisms insensitive to ammonium chloride.

376 INTRACELLULAR FATE OF SOLUBLE PROTEINS INTRODUCED INTO THE CYTOPLASM OF CELLS.
Fergus J. Doherty and R. John Mayer.Department of Biochemistry,University Medical School,Queen's Medical Centre, Nottingham NG7 2UH, U.K.
The fates of proteins erythrocyte-microinjected or scrape-loaded into 3T3-L1 fibroblasts have been studied.Bovine serum albumin (BSA),rabbit muscle,lactate dehydrogenase(LDH),pyruvate kinase(PK),and glyceraldehyde-3-phosphate dehydrogenase(G3PDH) radiolabelled with ^{125}I-iodine,were found in the cytosol (BSA) or in a cell fraction which,after homogenisation of injected cells,was coincident with DNA and cytoskeletal proteins on Nycodenz density gradients.LDH,PK and G3PDH wre degraded in injected cells with long half-lives(130-300h) and their degradation was inhibited by ammonium chloride,3-methyladenine and leupeptin. After introduction into cells LDH,PK,and G3PDH would not migrate into polyacrylamide gels unless previuosly boiled with 2-mercaptoethanol.The extracellular protein BSA was degraded rapidly after introduction into cells in a manner largely unaffected by inhibitors of lysosomal proteolysis, and was also 'exported' in large amounts to the medium.Endogenous cell proteins,pulse-labelled with radiolabelled amino-acid precursors, were found to accumulate in the cytoskeletal cell'residue' when chased in the prescence of leupeptin.These results are discussed in terms of the possibility of either a novel intermediate step in lysosomal proteolysis or the prescence of a cell 'dump' for proteins that cannot be immediately be degraded.

377 RELATIONSHIP BETWEEN PROTEIN TURNOVER AND CELL DEATH IN HIGH DENSITY 3T3 CELLS.
JS Amenta, SC Brocher, J. Mehta. Dept. of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, U.S.A.
3T3 cells were labeled for 5 days with ^{3}H-leu and ^{14}C-Tdr and chased in fresh growth medium without label for 24 hrs. Cells were then either maintained at high density or subcultured to low density to initiate rapid growth. Radioactivity in both the cell protein and DNA was assayed during a 4-5 day chase period. Cultures showing rapid growth showed a high rate of protein synthesis (approx. 0.80/d) and moderate rates of degradation (0.24/d). Label in the DNA decreased slowly, suggesting a low rate of cell loss during the growth period. As high densities were attained (40 µg protein)/cm$_2$, the cultures reached a plateau phase with little further growth. Plateau phase 3T3 cells showed low rates of protein synthesis, slightly higher rate of proteolysis and a high loss rate for label in the DNA. This pattern of protein and DNA degradation could be enhanced by (1) maintaining cells at a low density plateau phase with thymidine, or (2) maintaining selected cultures of 3T3 cells in rapid growth to extremely high densities. Control experiments with rat embryo fibroblasts did not show these phenomena; killing cells by freeze-thawing also did not show significant levels of proteolysis. These data suggest that at least in some 3T3 cell lines, growth inhibition is effected in part by an increased rate of cell death that is uniquely associated with proteolysis. The possibility of DNA turnover in these high density cultures is also considered.

378 T_3 EFFECT ON PROTEIN TURNOVER IN HEPATOCYTE PRIMARY CULTURES.
Gallo G.(1), Voci A.(1), Schwarze P.E.(2) and Fugassa E.(1).(1) Istituto di Fisiologia Generale-Università degli Studi-Genova.(2) Norsk Hydro's Institute for Cancer Research, Radiumhospitalet, Oslo, Norway.
Hepatocyte primary cultures have been extensively characterized with respect to regulation of protein synthesis and degradation. It has been demonstrated that cultured hepatocytes have a very low rate of protein synthesis(0.5-1%/h)as compared to rapidly proliferating cells (5-10%/h)and high capacity for bulk protein degradation along the autophagic-lysosomal pathway. Cultured hepatocytes lose protein and rapidly die unless amino acids are included in the medium at concentrations sufficient to block the autophagic-lysosomal pathway.Both the two processes which affect the protein level i.e. protein synthesis and protein degradation are target for biological regulation by serum and specific growth and hormonal factors. We have estimated protein synthetic rate in cultured hepatocytes by following the incorporation of 5 mM ^{14}C-valine into proteins for two hours. The level of incorporation was enhanced by 29-32% in cells cultured with 10^{-6}M T_3 for 5-6 days. Such an increase does not appear to be a reflection of alterations in the specific activity of the valine pool thus indicating an enhanced protein synthesis in T_3-cultured hepatocytes.
This result is consistent with the T_3-stimulated RNA synthesis in primary cultures of rat hepatocytes recently reported by us (Gallo G. et al.B.B.A.847, 140, 1985).In addition, our experiments demonstrate that protein degradation is increased in concert with protein synthesis, resulting in a greater rate of protein turnover in T_3-cultured hepatocytes. The underlying cellular mechanism of proteolytic action of T_3cannot be explained by our results obtained with the employment of inhibitors of intra-lysosomal proteolysis.

37° The role of actin and its reactions in the process of cell injury
Alexander Davidovich Brown, Tamara Pavlovna Mozhenok, Institute of Cytology, Academy of Sciences of the USSR, 194064 Leningrad, Tikhoretsky pr., 4, USSR.

Enhancement of light scattering, increase of viscosity, gelation, coagulation are the first and most significant signs of injury.
The data obtained indicates the essencial role in all substrate changes at injury G-and F-actin reactions such as polymerization, intraprotein interaction, denaturation. A number of nonelectrolytes (urea, glycerol, sucrose etc) prevent colloid reactions of injured cytoplasm and increase the resistance of cells to deleterious effects. According to our data, the same electrolytes inhibit G-actin polymerization.
Upon local injury of muscle fibre precipitate in formed locally exerting a protective effect. The proteinase inhibitors, -amino caproic acid in particular, inhibit the spreading of injury in muscle fibre.
A possibility to use actin reactions (polymerization, intraprotein interactions etc) for screening of substances that increase the cell resistance to injury is discussed.

380 4-METHYLUMBELLIFERYL-DERIVATIVE OF HEMOGLOBIN AS A NOVEL FLUOROGENIC SUBSTRATE FOR PROTEINASE ASSAY
Anahit Azaryan, Ya. Wozney, A. Galoyan. Institute of Biochemistry, P. Sevak str. 5/I, Yerevan 375044, USSR

A new fluorogenic substrate - hemoglobin-6-amidosuccinoyl-4-methylumbelliferone (4-MU-Hb) has been synthesized and characterized. A sensitive and inexpensive protease assay has been developed. Enzyme activity is estimated by measuring fluorescence of trichloracetic acid soluble 4-MU-conjugated peptides produced by proteolysis at 455 nm (excitation at 365 nm). The method satisfies the conditions for linearity of reaction velocity and incubation time and for proportionality with enzyme concentration. 4-MU-Hb was tested to determine activity of pepsin, papain as well as that of cathepsin B (EC 3.4.22.1), cathepsin D (EC 3.4.23.5) and high M_r aspartic protease, M_r=90K (EC 3.4.23.-) from human and bovine brain cortex. One unit of enzyme activity is defined as the amount of enzyme releasing 6-amidosuccinoyl-4-MU-peptide with fluorescence equal to that of 0.15 µmol of 6-amidosuccinoyl-4-MU (1h incubation at 37°C). Papain and pepsin can be measured at nanogram range; 1.5 µg of cathepsin D and 2 µg of cathepsin B produce a soluble fluorescence twice that of control under assay conditions, the sensitivity may be increased by longer incubation times such as 3 h. The $K_{M(appar.)}$ for the hydrolysis of 4-MU-Hb by cathepsin B and cathepsin D is estimated to be $4.6 \cdot 10^{-6}$ M and 10^{-6} M respectively. Thus, in contrast with 4-MU-casein, 4-MU-Hb may be utilized as a fluorogenic macromolecular substrate for both cysteine and aspartic proteases from different tissues. The assay is reproducible, has a low blank and uses Hb, that resembles natural substrates of most proteases.

381 ENERGY DEPENDENCE OF AUTOPHAGIC SEQUESTRATION
Paul B. Gordon*, Alfred J. Meijer** and Per O. Seglen*, *Department of Tissue Culture, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, 0310 Oslo 3, Norway, and **Section for Intermediary Metabolism, Laboratory of Biochemistry, University of Amsterdam, The Netherlands.

Autophagic sequestration has been measured in isolated rat hepatocytes by the use of radiolabelled sucrose as a probe. The sugar was injected into the cytosol by means of electropermeabilisation. Sucrose sequestration was virtually abolished under anoxic conditions, suggesting that this initial step in the autophagic pathway is completely energy-dependent. Respiration inhibitors like atractyloside and amytal depressed cellular ATP levels and reduced the rate of autophagic sequestration strongly. Glycerol, which specifically depletes cytosolic ATP, inhibited sequestration as well. A variety of other conditions also inhibited sequestration and ATP levels in parallel. The inhibitory effect of cycloheximide, previously known as a suppressor of autophagic-lysosomal protein degradation, could be fully accounted for by its reduction of cellular ATP. Since sequestration appears to be the rate-limiting, bioregulated step in the autophagic pathway, the known energy-dependence of autophagic-lysosomal protein degradation probably reflects the energy-dependence of the sequestration step.

382 INTRACELLULAR TRANSPORT OF ENDOCYTOSED GLYCOPROTEINS IN RAT LIVER PARENCHYMAL CELLS WITH HIGH AUTOPHAGIC ACTIVITY. Trond Berg, Grete Kindberg. Institute for Nutrition Research, School of Medicine, University of Oslo, Blindern 0316 Oslo 3,Norway.

The present study was undertaken to determine to what extent heterophagy and autophagy take place in the same lysosomes in rat liver parenchymal cells. Monolayers of rat hepatocytes were used. The cells were prepared by collagenase-perfusion of the liver. Autophagy was induced in the cells by incubation in a simple salt medium. The buoyant density of lysosomes in Nycodenz gradients changes when they transform into autolysosomes (autophagic vacuoles), and this was used to identify these vesicles. As a marker for the heterophagic process in the hepatocytes asialoorosomucoid was chosen. Asialoorosomucoid (AOM) was labeled with 125I-tyramine-cellobiose (125ITC). When 125ITC-AOM is degraded the labeled degradation products are trapped in the vesicles in which they are formed and they may therefore serve as markers for these organelles in cell fractionation experiments. By comparing the distributions of undegraded and degraded 125ITC-AOM with that of lysosomal enzymes in cells with high autophagic activity, it was found that degradation of 125ITC-AOM took place in a light subgroup of lysosomes. This subgroup probably also represented autolysosomes (autophagic vacuoles). The present data may suggest that the hepatocytes contain two subgroups of lysosomes, "active" and "passive" lysosomes. The "active" lysosomes are readily available ("fusogenic") for fusion with endosomes and autophagosomes; the "passive" lysosomes are recruited more slowly. In cells in coplete medium, in the absence of overt autophagy, the two types of lysosomes have about the same density in Nycodenz gradients. Following stimulation of autophagy the active lysosomes are rendered more buoyant in Nycodenz gradients.

383 EFFECT OF FASTING AND VINBLASTINE TREATMENT ON THE AUTOPHAGIC/LYSOSOMAL SYSTEM AND OTHER CELLULAR COMPONENTS OF MOUSE EXOCRINE PANCREATIC CELLS IN VIVO

L.László, A.L.Kovács, Magdolna Bajlinger, J.Kovács. Department of General Zoology,Eötvös Loránd University, H-1088 Budapest, Puskin u.3. Hungary

Autophagic vacuoles (AV) are very rare in normal exocrine pancreatic cells. However after treatment with the antimicrotubular drug vinblastine (VB) these cells contain large quantities of AV. Judging from the low level of AV, basal rate of autophagic segregation is to be very low in exocrine pancreatic cells. The huge increase in AV content after VB treatment may therefore derive in most part from direct or indirect stimulation of segregation by the drug. We investigated these possibilities in pancreatic acinar cells of mice which had been fasted for 24h, refed for 3h and fasted again for 2, 12, 24, 48, 72h. I.p. injection of 0.05 mg/g b.w. VB was given 0, 10, 22, 46 and 70h after the 3h feeding period. Fasting by itself did not elevate the cytoplasmic volume fraction of AV (measured by point counting morphometry) above 0.42%, the highest value of 72h starvation. The increase by VB treatment at 0 and 70h was as low as 0.57 and 0.75% respectively. Higher values were obtained at 10 and 46h (1.83 and 1.71% respectively), while the peak was reached at 22h being as high as 3.06%. These results somewhat unexpectedly show that like in liver cells, feeding conditions strongly influence the autophagic response of exocrine pancreatic cells after treatment with VB, The feeding status of the animal seems to play a permissive role for the increase of AV content upon VB administration. Fasting by itself elevates AV level only to a small extent. As the increase by VB can be very different albeit starting from the same control level, it seems that stimulation of autophagic segragation plays an important role in this process.

384 ISOLATION AND FUNCTIONAL CHARACTERIZATION OF AUTOPHAGIC VACUOLES FROM VINBLASTINE-TREATED PANCREAS USING A PERCOLL GRADIENT

E. Fellinger, G. Rèz, Gy. Pintèr, J. Kovács. Dept. of Gen. Zoology, ELTE Univ.,H-1088 Budapest, Puskin u. 3., Hungary

The predominant form of autophagy in pancreatic acinar cells is the isolation of cytoplasmic portions by a membrane pair of hitherto unidentified origin. Autophagosomes formed this way gain acid hydrolases only later, most probably by fusion with preexisting lysosomes. The drug vinblastine, disturbing this enzyme transfer, causes an accumulation of autophagosomes in many cell types, but most conspicuously in pancreatic exocrine cells. Therefore, we elaborated a cell fractionation method using 50% percoll gradient to purify autophagic vacuoles (AV) from pancreas of mice treated with vinblastine (50 mg/kg bw) for 4h. At this time cca. 10% of the cytoplasmic volume is segregated into AVs as shown by morphometry. Two AV fractions, both containing well recognizable organelle fragments were collected. AV I from the upper third and AV II from the bottom of the gradient. AV II, containing the smaller proportion of total AV recovered, showed about 5 times higher protein-based specific activity of the marker enzyme acid phosphatase than AV I. Both AV I and II showed morphologic signs of intravacuolar degradation, when incubated in 0.25M sucrose buffered with pH 7.4 citrate containing ATP. HPLC analysis of amino acids released to the medium from the vacuoles during incubation confirmed that intravacuolar proteolysis takes place in both AV I and II fractions.

385 EFFECT OF FASTING AND VINBLASTINE TREATMENT ON THE AUTOPHAGIC/LYSOSOMAL SYSTEM AND OTHER CELLULAR COMPONENTS OF MOUSE LIVER IN VIVO

A.L.Kovács, L.László, Gabriella Fiel, J.Kovács. Department of General Zoology, Eötvös Loránd University, H-1088 Budapest, Puskin u.3. Hungary

Previous results from several laboratories have shown that protein degradation as well as the amount of autophagic vacuoles (AV) in liver cells are influenced by feeding. The antimicrotubular agent vinblastine (VB) is known to produce considerable enlargement of the autophagic compartment. Stimulation of autophagic segregation or inhibition of the conversion of autophagosomes into autolysosomes have been considered as possible main mechanisms of the action of the drug. To get a better insight into the process we used mice which had been fasted for 24h and refed for 3h. The food then was withdrawn again and the animals were treated with 0.05 mg/g b.w. VB for 2h at 0, 10, 22, 46 and 70 hour of fasting. Cytoplasmic volume fraction of AV (measured by point counting morphometry) steadily increased during fasting, starting from 0.02% immediately after refeeding and reaching the highest 1.24% value in the 72h fasted cells. The increase of the amount of AV by VB treatment was lowest (0.21%) when the drug was given immediately after feeding, it was higher at 10h (0.40%) and 22h (0.34%), highest at 46h (0.85%) and lower again at 70h (0.74%). Accumulation of secretory vacuoles (VLDL) after VB treatment was very conspicuous in early periods of fasting (0h, 10h, 22h) but showed a decline in 46h and 70h fasted animals. The results of our experiments favour the conclusion that the effect of VB treatment is secondary to factors determining the physiological status of the animals.

386 REGRESSION OF AUTOPHAGIC VACUOLE COMPARTMENT AFTER CYCLOHEXIMIDE TREATMENT OF MOUSE PANCREATIC ACINAR CELLS UNDER THE INFLUENCE OF VINBLASTINE

G.Réz, E.Fellinger, Z.Pálfia, J.Kovács. Dept.of Gen.Zoology, ELTE Univ., H-1088 Budapest, Puskin u. 3, Hungary

Vinblastine (VB) is known to cause an expansion of autophagic vacuole (AV) compartment in many cell types. As we measured by morphometry, a single injection of the drug (10mg/kg bw) caused a rapid increase in cytoplasmic volume fraction of total AVs reaching 0.6, 1.9, 2.9, 6.7, 11.5, 13.1, 11.64, and 12.5% at 1,2,3,4,6, 7, 24, and 25hours after VB, respectively. Thus, AV compartment extends rapidly in the first 7 postinjectional hours and remains on a high level for a long time after that. Cycloheximide (CH) is known to block autophagy by inhibiting the formation of AVs. When mice were given this drug (0.2mg/g bw) for 1h, 1, 3, 6, and 24h after VB administration, the volume fraction of total AVs were 0.3, 1.35, 7.4, and 5.5% respectively. These findings show that 50, 53.4, 35.6, and 52.8% of the volume of the total AV compartment regressed, when the cells were under the influence of CH during the 2nd, 4th, 7th and 25th hours after VB, respectively. The results indicate, that the turnover rate of AV compartment in VB-treated pancreatic acinar cells is very slow compared to the 5-10min half lives of AVs measured in other cell types under normal or close to normal conditions. On the other hand, since the volume of the total AV compartment is very large in the VB-treated pancreas after the 6th hour, the amount of cytoplasm degraded under such circumstances is considerably high.

387 DOSE DEPENDENT STIMULATION OF CELLULAR AUTOPHAGY BY VINBLASTINE APPLIED LOCALLY TO THE RAT LIVER SURFACE IN SITU.

Ulrich Pfeifer and Thomas Hofbauer
Department of Pathology, University of Würzburg, D-8700 Würzburg, FRG

The mechanism leading to the expansion of the compartment of autophagic vacuoles in the rat liver after systemic application of vinblastine is still a matter of debate. In order to avoid systemic effects, vinblastine was applied for 20 min at different concentrations (10^{-5}M, 10^{-7}M, and 10^{-9}M) to the surface of rat liver in situ by use of small plastic rings, as described elsewhere (Lab Invest 50.348-354,1984). Three different types of vacuoles were distinguished in the electron microscopical evaluation: Early autophagic vacuoles containing cytoplasmic components without any structural alteration (type I), late autophagic vacuoles containing partly degraded cytoplasm (type II), and vacuoles containing debris which could no longer be identified (type III). Compared to control areas exposed to NaCl only, vinblastine increased the volume fraction of all three types of vacuoles. The increase of type I, II, and III was 3.0, 2.1, and 1.5-fold at 10^{-5}M, 2.6, 2.0, and 1.3-fold at 10^{-7}M, but 2.2, 1.7, and 1.0-fold at 10^{-9}M, respectively. The size distribution of type I profiles containing cytoplasmic ground substance together with ribosomes and ER or Golgi membranes, was significantly shifted towards larger profiles under the influence of vinblastine. These data suggest that the increase in number and fractional volume of autophagic vacuoles induced by vinblastine is not merely the consequence of accumulation due to inhibited autophagosome lysosome fusion, but reflects true and direct stimulation of the autophagic lysosomal pathway.

388 AUTOPHAGIC SEQUESTRATION IN HEPATOCYTES

Per O. Seglen, Henrik Høyvik and Paul B. Gordon, Department of Tissue Culture, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, 0310 Oslo 3, Norway

The intracellular sequestration of hydrolysable (lactose) and non-hydrolysable (sucrose, raffinose) radiolabelled sugars, introduced into the cytosol by means of electropermeabilisation, has been used to characterise the organelles involved in hepatocytic autophagy. Raffinose, a trisaccharide, is sequestered only by the autophagic-lysosomal system, whereas the disaccharides sucrose and lactose are also taken up non-autophagically, by the mitochondria. The density gradient distribution of raffinose is initially heterogeneous, indicating its presence both in lysosomes and in pre-lysosomal vacuoles. This distributional heterogeneity can furthermore be demonstrated by altering lysosomal density with agents like Triton WR-1339 and leupeptin.

Sequestered lactose is rapidly hydrolysed in lysosomes, but accumulates in pre-lysosomal vacuoles if fusion with lysosomes is inhibited by the drug vinblastine. Sucrose, on the other hand, is normally not hydrolysed, but if the hepatocytes are allowed to endocytose the enzyme invertase, a gradual disappearance of sequestered sucrose from the lysosomes can be observed. The experiment demonstrates convergence of the autophagic and endocytic pathways, and suggests a strategy for analysis of individual steps in these pathways.

389 ANALYSIS OF VINBLASTINE INDUCED AUTOPHAGOCYTOSIS IN MOUSE PAROTID CELLS.

M. Sterle, N. Pipan, K. Jezernik. Institute of Human Biology, Medical Faculty, Lipiceva 2, 61000 Ljubljana, Yugoslavia

The origin of the membrane of newly formed autophagic vacuole (AV) was studied in mouse parotid cells treated with vinblastine (VBL). Special attention was pard to the comparison of the membranes of the Golgi apparatus (GA) and granular endoplasmic reticulum (GER) to the membrane of AV, respectively. Autophagocytosis was induced by an intraperitoneal injection of VBL (100 mg/kg b.w.). Unbuffered Os0 was used as the marker for cis Golgi cisternae, trans cisternae were characterized by thiamine pyrophosphatase (TPP-ase) and acid phosphatase (AcP-ase) activity. The numerous AV's comprise most frequently GER and mitochondria. The limiting membranes of fast all newly formed AV's are always covered with ribosomes. The space between the limiting membranes of the newly formed AV's shows neither deposits of the unbuffered OsO_4 nor TPP-ase and AcP-ase activity. The presence of ribosomes and the lack of the enzyme activity on the limiting membrane of newly formed AV's suggests that their limiting membranes can originate from GER.

390 STIMULATION BY PROPRANOLOL AND VERAPAMIL OF CELLULAR AUTOPHAGY IN THE RAT MYOCARDIUM

Marcel Bahro and Ulrich Pfeifer, Department of Pathology, University of Würzburg, Luitpoldkrankenhaus, Joseph-Schneider-Str. 2, D-8700 Würzburg, Fed. Rep. Germ.

The influence on myocardial cellular autophagy of two cardiodepressant drugs differing in their pharmacological action was examined in adult male Sprague - Dawley rats (bd. wt. 270 - 360 gm). In the first experimental series 10 rats were given the beta-blocking agent propranolol (3mg/kg bd. wt., s.c.), and 10 control animals recived physiological saline. In the second series 8 animals were treated with the calcium-antagonist verapamil (9 mg/kg bd. wt., s.c.), and 8 control animals with physiological saline. The injections were given at 6 p.m., shortly before switching from light to dark, i.e. the time interval of the diurnal minimum of cellular autophagy. 2 to 4 hs after the injections the animals were killed by retrograde perfusion via the abdominal aorta with a paraformaldehyde-glutaraldehyde mixture. Cubes of myocardium from the left anterior wall were processed for electron microscopy and were morphometrically evaluated for volume fraction and numerical density of early stages of autophagic vacuoles (AV). Propranolol and verapamil increased the AV volume fraction 4.3 and 2.7-fold, and the numerical density 2.3 and 1.7-fold, respectively. Since verapamil affects neither the beta-adrenoreceptor nor the intracellular levels of the second messenger cAMP, the only common denominator for the stimulation of cellular autophagy seems to be the cardiodepressant effect of the both agents. The data suggest that the increase in cellular autophagy is an early regulatory step in the adaption of myocardial mass to reduced work load.

391 LYSOSOMOTROPIC AGENTS, ENDOCYTOSIS AND AUTOPHAGY.

Korolenko T.A. Central Research Laboratory, Novosibirsk Med. Inst. Novosibirsk, USSR

Lysosomotropic agents are taken up selectively into lysosomes during in vivo administration and cause the specific changes of structure and functions of these particles. The rate of adsorptive and fluid endocytosis, physico-chemical and electron microscopically detected structural changes of rat liver lysosomes were studied during administration of two kinds of lysosomotropic agents: suppressing the rate of intralysosomal proteolysis (suramin, gold sodium thiomalate, chloroquine) and not influencing on this process (triton WR 1339, PVP). Overloaded rat liver cells were able to uptake other lysosomotropic agents by adsorptive and fluid endocytosis. Administration of triton WR 1339 (85 mg/100 g. of body weight, 24,48 h) led to formation of autophagolysosomes; autophagy increase was observed also during suramin administration (250 mg/kg, 24,48 h). The reasons of autophagy increase possibly were different in case of suramin (disturbance of fusion processes, suppression of the intralysosomal proteolysis rate and changes of period of half-life of autophagic vacuoles) and triton WR 1339 administration (parallel increasing of rate of heterophagy and autophagy?). Accumulation of lysosomotropic agents in rat liver cells was followed by different degrees of lysosome's labilization, that was connected with lysosome overloading and formation of autophagic vacuoles, which membranes had changed structural features.

392 RELATIONSHIP BETWEEN PROTEOLYTIC ACTIVITIES IN MUSCLE IN VITRO AND GROWTH OF PIG AND CATTLE

Rosemarie Weikard, Berthold Löhrke. Academy of Agricultural Sciences, Research Institute of Animal Production Dummerstorf-Rostock, Dept. Genetics, 2551 Dummerstorf, GDR

Parameters of intracellular proteolysis in muscle homogenates of pig (p) and cattle (c) measured in the azocasein-assay (pH 6,9, 1 mM Ca^{2+}) at an early developmental stage of the animal are related to characteristics of growth (linear regression analysis).
There were find out correlations of 0,7 (p) resp. 0,3 (c) between proteolytic parameters and expenditure of food energy per unit gain and -0,6 between daily weight gain and protein degradative activities in muscle homogenates of pigs.

393 ULTRASTRUCTURAL ASPECTS OF EU- AND PROKARYOTIC CELL INTERACTIONS IN LEAFHOPPERS

Toomas Tiivel. Institute of Chemical Physics and Biophysics, Estonian Academy of Sciences, Tallinn, Akadeemia tee 23, Estonia 200026 USSR

Specialized cells of leafhoppers *Aphrophora alni* Fall. and *Philaenus spumarius* L. (*Insecta; Homoptera*) containing endocytobiotic microorganisms (E), were studied electronmicroscopically. Three types of E were found, all being surrounded by a cytoplasmic membrane, a membraneous wall typical of gram-negative bacteria, and, on the outside, by an additional membraneous casing, most probably a derivative of the bacteriocyte, which forms a kind of space around each E - a bacteriophoric vacuole. Through these structures the interconnections (metabolic and probably also genetic) of cells participating in this coexistence, are realized. The vacuoles considerably differ in size, beginning with a slit-like space between two cells up to a rather big vacuole, the size of which may exceed the size of E by some microns. In many cases and in all the described types of E fine granular substrates as well as vesicules and membraneous formations were found in these vacuoles. From the outer surface of E, membranes exfoliated and were twisted into round figures, or formed a border structure of 5-6 -layer coats around the cytoplasm of the E. The accumulation of new membranes occurred around E as well as around concentric figures between them. There were no such myelin-like organized structures in the cytoplasm of the neighbouring cells of the bacteriocytes, which shows their specificity. The ultrastructural peculiarities of the bacteriophoric vacuoles indicate their functional activity in such interconnections of pro- and eukaryotic cells. We may regard the exfoliation of membranes and the formation of specific myelin-like figures as some evidence of the fact that the host is probably able to regulate the number of E by digesting them and, in this way, getting the necessary nutritive substances for itself, leaving the residual bodies (consisting mainly of lipoproteins) in the bacteriocytes. Another possibility is that these structures represent some kind of intracellular depot of certain substances by the insect, which are secreted by E.

394 TARGET PROTEINS FOR SECOND MESSENGERS IN THE NERVOUS SYSTEM. P. De Camilli, CNR Center of Cytopharmacology and Dept. of Medical Pharmacology, Univ. of Milano, Via Vanvitelli 32, Milano Italy.

The integrated function of neurons in the nervous system involves a continued exchange of information between the neuronal surface and the neuronal cytoplasm. The neuronal surface is the site involved in the generation of electrical signals (via ligand-regulated ion channels) and in their passive or regenerative spreading (the latter via voltage-regulated ion channels). The electrical properties of the cell surface, however, are continuously modulated by intracellular 2nd messengers which are generated either by the electrical signals themselves (for example via voltage-gated Ca^{2+} channels) or by receptors for neurotransmitters and neurohormones coupled to 2nd messenger-generating systems. Intracellular 2nd messengers can affect surface properties in a variety of ways which range from ion channel regulation to change in the geometry of neuronal processes. Furthermore, intracellular 2nd messengers are involved in transducing electrical signals into a secretory response in nerve endings. The precise mechanisms by which 2nd messenger signals are transduced in physiological effects are to a large extent still unknown but they are now starting to be unraveled. A rather general mechanism of action of 2nd messengers appears to be the reversible modification of key regulatory proteins by reactions of phophorylation-dephosphorylation, catalyzed by 2nd messenger-dependent protein kinases and protein phosphotases. The 2nd messenger-receptor protein can be either the enzyme itself or one of its regulatory subunits. A large number of processes and proteins in neurons have already been shown to be regulated by phosphorylation-dephosphorylation. In fact, protein phosphorylation seems to be such an important mechanism of regulation that identification of major phosphoproteins regulated by 2nd messengers has already proved to be a way to gain insight into poorly understood neuronal processes.

The particular contribution of our laboratory to the field has been to study some aspects of brain phosphorylation systems in situ. This work constitutes an important complement to the more classical biochemical studies in the field because it allows to relate biochemical findings (often obtained in cell-free systems) to physiological processes occurring in vivo. Furthermore, this work has been useful to give us some clues on the function of proteins previously simply known as bands in gels, and information on previously unknown sites of action of 2nd messengers. An important finding toward which our work has contributed is that protein phosphorylation systems in different neurons exhibit many common features but also important specificities.

Many major phosphoproteins are common to all neurons. These include all those proteins that are important in basic neuronal functions. For example, an important common site of regulation in dendrites appears to be the dendritic cytoskeleton via MAP2. MAP2, a microtubule-associated protein which is selectively concentrated on dendritic microtubules, is phosphorylated at multiple sites by a variety of protein kinases including cAMP-dependent and Ca^{2+}/calmodulin dependent protein kinases. It is thought to crosslink microtubules to other cellular structures and it may be involved in mediating action of 2nd messengers (generated at axodendritic synapses) on the structural properties of dendrites. Such actions may play a role in dendritic growth and plasticity. A key target site for axon terminal regulation is Synapsin I. The latter is a nerve cell-specific phosphoprotein also phosphorylated at multiple sites by cAMP-dependent and Ca^{2+}/calmodulin-dependent protein kinases. It is a cytoplasmic peripheral membrane protein of the membrane of synaptic vesicles which appears to be involved in crosslinking the vesicle surface to the cytoskeleton. It is selectively associated with small vesicles (i.e., vesicles involved in the release of classical neurotransmitter only) and not with large dense-core vesicles, i.e., secretory organelles storing neuropeptides. Its phosporylation by 2nd messengers appears to have a regulatory role on the release of classical neurotransmitters.

In spite of the presence of these common phosphoproteins in all neurons, protein kinases and protein phophatases, including those by which Synapsin I and MAP2 are phosphorylated, are present at very variable concentrations in different families of neurons and some appear to be almost specific for certain neuronal classes. This variability, which may reflect variability in synaptic inputs, suggests that phosphoproteins present in all neurons may be regulated by different 2nd messenger systems in different neurons. This can be accomplished in several ways. For example, the same phosphoprotein can be phosphorylated at the same site or at distinct sites by distinct protein kinases. Also, it is possible for the same phosphoprotein to be regulated either by a 2nd messenger via a protein kinase or by another 2nd messenger via regulation of a protein phosphatase [Nestler, Walaas and Greengard, Science, 225, 1357 (1984)].

Some protein kinases are distributed unevenly in the cell and their localization can give us clues about unknown sites of action of 2nd messengers. One such example is the finding of a high concentration of RII (the regulatory subunit of type II cAMP-dependent protein kinase) in the Golgi region, and, in particular, in the trans-Golgi region. Phosphorylated substrates in the Golgi region are still unknown, but indirect evidence suggests that they may be microtubule-associated proteins. The trans-Golgi region is the headquarters of intracellular traffic. Second messengers by controlling microtubule function in this region may control a neuronal function in distal compartments by regulating vesicle traffic in and out of the Golgi. Furthermore, as suggested by a recent report (Nigg et al, EMBO J. 4, 2801, 1985), concentration of RII in the Golgi area may be a way to maintain a high concentration of the catalytic subunit of the kinase in proximity of cell nuclei, and therefore to provide a mechanism by which cAMP can regulate nuclear function. [Work supported in part by grants from MDA, and from the Italian National Research Council (grant no. 8303600 and 8402274) to PDC]

395 PROTEIN PHOSPHORYLATION IN CELL REGULATION. ORD, M.G. AND STOCKEN, L.A., DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF OXFORD, SOUTH PARKS ROAD, OXFORD, OX1 3QU.

The 25 years since the discovery of cyclic AMP-dependent protein kinase have seen many dramatic developments in our understanding of the importance of protein phosphorylation in cell regulation:

1) The discovery of the Ca^{2+}/calmodulin regulated systems which with cyclic AMP-dependent kinase offers a potential for temporarily and spatially controlled phosphorylation.

2) Multisite phosphorylation of key enzymes with interdependent regulation of the different kinases and phosphatases involved.

3) The discovery of the Ca^{2+} activated, membrane-associated, protein kinase C, with its potential for modifying membrane functions such as hormone receptors or solute transporters.

4) The phosphoinositide dual control mechanism, affecting both protein kinase C and intracellular Ca^{2+} regulated systems.

5) The role protein phosphorylation plays in central neurotransmission.

6) The finding in plants of regulation of protein phosphorylation by redox systems.

Unresolved problems in seryl protein phosphorylation include the significance and regulation of the less well characterised kinases casein kinases I and II, with their capacity to phosphorylate serine adjacent to acidic residues. Also the biological relevance of phosphorylation of structural proteins such as the ribosomal protein 6, or the many nuclear proteins whose function is still unknown. The increasing availability of protein kinase -ve cells opens a way into these problems.

The discovery of tyrosine protein kinase and its involvement in oncogenesis has stimulated the search for specific control points with the cell cycle for which tryosine phosphorylation is uniquely required. Whilst the validity of this concept for normal cell growth has still to be established, the infrequency of tyrosine residues in proteins suggest a precise on/off control contrasting with the incremental response possible through stepwise phosphorylation of the commoner serine residues.

396 METABOLISM AND SECOND MESSENGER ROLES OF INOSITOL PHOSPHATES.
R.F. Irvine, AFRC Institute of Animal Physiology, Babraham, Cambridge, CB2 4AT, UK.

Stimulation of tissues with many neurotransmitters, hormones or growth factors causes an increase in the turnover of a group of lipids in the cell membrane known as the inositides. The principal stimulated reaction is the phosphodiesteratic hydrolysis of phosphatidylinositol(4,5)bisphosphate, with the resulting formation of two compounds, diacylglycerol and inositol(1,4,5)trisphosphate. The former probably remains in the membrane, and stimulates the activity of a protein kinase called protein kinase C. The latter compound, Ins(1,4,5)$\underline{P}_3$, is released into the cytosol. Present evidence strongly supports the hypothesis that it binds to a receptor on the endoplasmic reticulum, and this binding opens a pore in the e.r. to release Ca^{2+} into the cytosol. The Ca^{2+} rise which ensues stimulates Ca^{2+}-dependent protein kinases and other Ca^{2+}-controlled processes in the cytosol.

Ins(1,4,5)$\underline{P}_3$ is inactivated by a 5-phosphatase which forms Ins(1,4)$\underline{P}_2$, a compound which does not mobilize Ca^{2+}. Recently we have uncovered another, alternative, route of metabolism of Ins(1,4,5)$\underline{P}_3$ which is in some tissues quantitatively as important as dephosphorylation. This alternative route begins with a novel Ins(1,4,5)$\underline{P}_3$-kinase which phosphorylates Ins(1,4,5)$\underline{P}_3$ to yield inositol(1,3,4,5) tetrakisphosphate. This Ins(1,3,4,5)$\underline{P}_4$ is then dephosphorylated (by the same 5-phosphatase as that which hydrolyses Ins(1,4,5)$\underline{P}_3$) to give Ins(1,3,4)$\underline{P}_3$; the latter compound is then further catabolized by an, as yet unknown, route.

The evidence for the second messenger role of Ins(1,4,5)$\underline{P}_3$ will be discussed, as will details of its mode of action. Also to be discussed will be the function of the tris/tetrakis phosphate pathway, and whether any second messenger role of Ins(1,3,4,5)$\underline{P}_4$ or Ins(1,3,4)$\underline{P}_3$ is likely.

397 *PROTEIN KINASE C- STRUCTURAL AND FUNCTIONAL ANALYSIS.*

S. Stabel, S. Young, R. M. Marais, P. J. King, M. D. Waterfield, L. Coussens, T.J. Dull*, A. Ullrich* and P. J. Parker. Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, U.K. *Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080, U. S. A.*

Protein kinase C is involved in cellular growth control through transduction and modulation of extracellular signals. The protein has been shown to be the major cellular receptor for tumour promoting phorbol esters suggesting a correlation between the central role of protein kinase C in signal transduction from physiological stimuli and the pleiotropic effects of phorbol esters on cellular growth control.

In order to elucidate the structure of protein kinase C and to analyse its role in the control of cellular proliferation we have purified the enzyme from bovine brain and obtained approximately 100 residues of amino acid sequence from peptide fragments. Based on this sequence oligonucleotides were synthesised and used to identify a number of cDNA clones coding for bovine protein kinase C. From the cDNA sequence the amino acid sequence of protein kinase C has been deduced.

The cDNA clones have provided probes to analyse the expression of protein kinase C in normal and neoplastic tissue.

In addition we have generated a series of antisera to defined regions of protein kinase C in order to investigate the function, expression and localisation of the enzyme.

398 THE POSSIBLE IDENTITY OF HISTONE KINASE II AND A PROTEOLYTIC FRAGMENT OF PROTEIN KINASE C

Anna Faragó, T. Romhányi, J. Seprődi, F. Antoni
1st Institute of Biochemistry, Semmelweis University Medical School, P.O.B. 260, 1444 Budapest 8, Hungary

The protein kinase referred to as histone kinase II (HK II) has been characterized by the property to phosphorylate in vitro the serine residue beside the single phenylalanine residue of calf thymus H1 histone at variance with the cAMP-dependent protein kinase which phosphorylates the Ser-37 residue. We have described a cyclic nucleotide independent histone kinase which has been identified as HK II. A synthetic oligopeptide substrate has been designed for this protein kinase. The analysis of the amino acid sequences of H1 and H2b histones around the sites phosphorylated by HK II and the ability of this enzyme to phosphorylate certain oligopeptides have shown that HK II recognizes the Ser-Xaa-Lys sequence. The oligopeptide Ala-Ala-Ala-Ser-Phe-Lys-Ala-Lys-Lys-amide has been proved to be a specific substrate for HK II and it has been used as a tool to demonstrate the presence of HK II in the extracts of different tissues. HK II purified from calf thymus has an apparent molecular mass about 55 kDa but several observations suggest that it may be a proteolytic fragment of a larger molecule. It is known that the Ca^{2+}/phospholipid dependent protein kinase (protein kinase C) can be activated irreversibly by partial proteolysis and it is conceivable that under certain conditions the proteolytic derivate of protein kinase C may function within the cell. Several features of HK II (substrate specificity, chomatographic behaviour, occurence in different tissues) are very similar to those of protein kinase C. This similarity between the two enzymes strongly supports the presumption that HK II is a proteolytic fragment of protein kinase C.

399 TRANSFORMATION OF CELLS BY PROTEIN-TYROSINE KINASES. J. Gordon Foulkes, Lab. Eukaryotic Molecular Genetics, National Institute for Medical Research, Mill Hill, London, NW7 1AA, England.

Although originally defined by analysis of acutely transforming retroviruses, it is now apparent that the genomes of all eukaryotic species contain a set of genes capable of transforming cells to the malignant state. Around 40 transforming genes, termed oncogenes, have been isolated so far. Regardless of mechanism of proto-oncogene activation, the most important question for our laboratory is how a single gene, and hence a single protein, can actually transform cells? This clearly necessitates an understanding of the biochemistry of transforming proteins. Since the discovery of the cAMP-dependent protein kinase in 1968, reversible protein phosphorylation is now established as the major mechanism for the regulation of protein function in eukaryotic cells. The first evidence that oncogenes might directly encode protein kinases originated from work described in Erikson's laboratory, for the transforming protein of the Rous sarcoma virus, pp60src. Shortly after this discovery, Hunter and Sefton identified tyrosine as the novel phosphate acceptor. Phospho-tyrosine is a rare modification in the normal cell (0.02% of the total phosphoamino acids) but it is implicated as having an important role in the regulation of cell growth by a number of recent findings. First, half of all known oncogenes have now been shown to encode protein-tyrosine kinase activities. Second, the plasma membrane receptors for several growth stimulating hormones, namely EGF, PDGF, IGF-1, CSF-1 and insulin, have been demonstrated to be associated with protein-tyrosine kinases. Third, the oncogene of the Simian sarcoma virus, sis is derived from PDGF, the oncogene erb-B is a truncated form of the EGF receptor, while fms has recently been shown to be related to the CSF-1 receptor. Thus, protein-tyrosine kinases appear to be essential components in the regulation of both normal and abnormal cell growth.
Transformation may result from an overexpression of the kinase activity. This would increase the

degree of phosphorylation of proteins which are substrates for the kinase encoded by the related proto-oncogene. An alternative model is to suggest that mutations alter the protein kinase specifity thus leading to modification of proteins or sites which are not phosphorylated by the normal enzyme. In either scheme transformation involving protein phosphorylation should be envisaged as a dynamic equilibrium process, the balance of the kinase-phosphatase activities. To date, little is known about phosphotyrosyl-protein phosphatases but there appear to be multiple forms, all of which are distinct from the better characterised phosphoseryl-phosphothreonyl phosphatases.

To understand how protein-tyrosine kinases regulate cell growth necessitates the identification of their substrates. To date, however, no targets have been firmly established for any protein-tyrosine kinase. My laboratory is currently attempting to identify physiological targets of the v-abl kinase. Previously we have defined the minimal size v-abl gene capable of cell transformation. Using an expression vector system in E.coli we have succeeded in purifying the corresponding protein to homogeneity. Some problems with prokaryotic expression vector systems will be discussed. Having obtained a highly active purified protein-tyrosine kinase, we can now test possible candidate target proteins as substrates in vitro. If stoichometric phosphorylation is obtained, one can then look for changes in the target protein's activity. If such changes are observed, however, one must then look in vivo for the corresponding phosphorylation induced activity change. In vitro data alone can be very misleading. An example of such artifacts will be presented with respect to the enzyme topoisomerase I.

Although all protein-tyrosine kinases appear to be specific for tyrosine residues in vitro, the addition of epidermal growth factor, platelet-derived growth factor or insulin to responsive cells also results in the increased phosphorylation of certain proteins on serine residues. Similarly, in cells transformed by A-MuLV or RSV, both of which encode protein-tyrosine kinases, an increase in protein-bound phosphoserine has been observed. Among these phosphoseryl-proteins, ribosomal protein S6 is of particular interest because its phosphorylation is correlated with growth promoting stimuli in a wide variety of systems.

Previously, we had observed that the phorbol ester, TPA, which is known to activate protein kinase C and induce S6 phosphorylation, induces the phosphorylation of the same five S6 phosphopeptides as found in Abelson transformed cells. We have, therefore, tested a variety of models whereby the Abelson kinase might regulate S6 phosphorylation by activation of protein kinase C. Our data indicates that the phosphoinositide pathway is constitutively activated following transformation by A-MuLV. This could lead to a sustained increase in the steady state concentrations of both diacylglycerol, leading to the activation of protein kinase C, and inositol trisphosphate, leading to the release of intracellular calcium. Other phosphoinositide metabolites, eg. arachidonic acid, might also act on systems potentially important in the transformation process. This work suggests that a valuable approach in studying the mechanism of cell transformation by protein-tyrosine kinases will be to identify the physiological substrates of both protein kinase C and the Ca^{2+}-calmodulin dependent kinases and phosphatases. Our most recent results on the mechanisms whereby protein-tyrosine kinases stimulate PI turnover will be presented.

I will then briefly review the autocrine hypothesis of Spron and Todaro which proposed that transformed cells produce transforming growth factors (TGFs) which are released to stimulate the growth of the cells that produce them. Although Abelson transformed cells produce high levels of αTGFs, I will present evidence which strongly suggests that αTGFs are not essential for in vitro transformation of cells by the Abelson kinase.

Finally, I will conclude the talk by outlining how an understanding of the association between protein phosphorylation and transformation should lead to a number of novel approaches with regards to cancer chemotherapy.

400 CASEIN KINASE-2: ITS SPECIFICITY AND POSSIBLE ROLES IN CELL REGULATION.

Lorenzo A. Pinna and Flavio Meggio. Institute of Biological Chemistry, University of Padova, Via F. Marzolo 3, 35131 Padova, Italy.

The term "casein kinase" is commonly applied to widespread protein kinases operationally defined after their sharp preference in vitro for casein over histones as artificial substrate, but quite distinct from the mammary gland enzyme(s) resposible for the in vivo phosphorylation of casein. Such ubiquitous casein kinases are Ser/Thr-specific and independent of cyclic nucleotides, Ca^{2+}, diacylglicerol and any known effectors of other protein kinases. Furthermore casein kinases of the 2nd type (CK-2, termed also G or TS) can use GTP besides ATP as phosphate donor, affect mostly Thr residues of casein fractions and are extremely sensitive to heparin inhibition. Although oligomericity appears to be a common feature of all known CK-2, their quaternary structure seems to be different in animals, where hetrotetramers $\alpha_2\beta_2$ or $\alpha\alpha'\beta_2$ have been constantly described, and in yeast and moulds whose CK-2 are lacking the low Mr, non catalytic, β-subunits. Such a sharp difference in quaternary structure doesn't alter the site specificity of CK-2 which is always determined by a cluster of acidic residues close to the C terminal side of the target aminoacids, as disclosed by both inspecting their phosphorylation sites and using synthetic peptide substrates with variable sequences and aminoacid composition. Rather, the presence of the 25 kDa autophosphorylatable β-subunits may confer to animal CK-2 a special susceptibility to polycationic effectors, like protamines and polylysine, which lower by one order of magnitude the Km values for the protein substrates while preventing at the same time the autophopshorylation of the enzyme at its β-subunit. Since a class

of polycation stimulated protein phosphatases (PCS-phosphatases) has been also detected, a cyclic modulation of animal CK-2 activity through autophopshorylation and PCS-phosphatase mediated dephosphorylation, coordinately regulated by polybasic effectors, has been proposed.

The growing list of potential targets of CK-2 already includes key enzymes of glycogen and lipid metabolism (e.g. glycogen synthase and acetyl-CoA-carboxylase), many protein factors involved in the regulation of gene expression, cell proliferation and protein synthesis, and the regulatory subunits of both cAMP-dependent protein kinase (type-II) and ATP, Mg^{2+}-dependent protein phosphatase (type-1). In some cases CK-2 exerts a synergetic effect on subsequent phosphorylation by another protein kinase termed F_A or GSK-3 which in turn inhibits glycogen synthase and activates protein phosphatase (type-1). Altogether these data point to a central role of CK-2 in the coordination of the cellular network of protein phospho/dephosphorylations controlling so many and apparently unrelated biological processes.

In rat liver cytoplasm CK-2 participates into at least two distinct multimolecular aggregates: glycogen particles, where it can phosphorylate glycogen synthase, and a soluble complex where it is associated with a 90 kDa phosphoprotein, representing one of its best substrates. The identification of this 90 kDa target of CK-2 with the 90 kDa heat shock protein which copurifies with the glucocorticoid receptor (E.R. Sanchez et al., J. Biol. Chem. 260, 12398 (1985)) and interacts with the $pp60^{src}$ tyrosine specific protein kinase (H. Oppermann et al., Proc. Natl. Acad. Sci USA 78, 1067 (1981)) is being explored.

401 CYCLIC AMP AND THE REGULATION OF THE EUCARYOTIC GENOME EXPRESSION.

E.S.Severin, M.V.Nesterova. Institute of Molecular Biology, Academy of Sciences of USSR, Moscow B-334, USSR.

The fact that cAMP-dependent protein kinase activation induces RNA synthesis raises a question about biopolymers synthesis regulation by cyclic nucleotides at the molecular level.We tried to find out mechanism of protein kinase subunits interaction with structural elements of the genome, to identify nuclear acceptor sites for the enzyme and to elucidate biological role of this interaction for the cell.

Data are presented that cAMP-dependent protein kinase catalytic subunit is able to associate with gomologous DNA. Regulatory subunit appears to interact with nuclear proteins.Among them histone H1 and several acidic proteins were identified.

Protein kinase subunit introduction into cells of different types revealed these proteins to influence cell proliferation and RNA-,DNA- and protein synthesis.

Regulatory subunit translocation into 3T3 cell nuclei is found to effect protein synthesis. Originally the cells synthesise protein P15 with molecular mass 15000Da and isoelectric point 6,3.New technique has been developed for isolation of protein P15 in homogeneous state. Protein P15 appears to take part in regulation of cell proliferation. Possibly, P15 acts like known growth factors.

A scheme is proposed to describe cell function of cAMP-dependent protein kinase type II.The scheme points out a role of cAMP binding by regulatory subunit and cAMP-dependent phosphorylation in control of eucaryotic cell genetic activity.

402 CELLULAR AND EXTRACELLULAR SUBSTRATES OF CELL SURFACE PROTEIN KINASE.

Dieter Kübler, Olga Bill, Michael Fehst, Walter Pyerin, Erika Burow, Volker Kinzel.
Institute of Experimental Pathology, German Cancer Research Center, Im Neuenheimer Feld 280, D-6900 Heidelberg, FRG

A cell surface located protein kinase (PK) activity with properties of cyclic nucleotide-independent types including the phosphorylation of serine and threonine residues, has been characterized on intact cultured cells and in blood cells (J.B.C. 257, 322 (1982)). Such ecto-kinase can be liberated from intact cells into their extracellular fluid by substrate (PNAS 80, 4021 (1982)). Recently the same type of kinase activity was detected in blood plasma and serum (BBRC 133, 8 (1986)).

Ecto-kinase activities are directed towards cell surface located proteins as well as extracellular substrate proteins. With different cell types characteristic endogenous labeling pattern were generated. However, there are clearly some similar predominantly labeled polypeptides. The surface labeled proteins partly characterized as glycoproteins were easily removed by mild trypsinization. Studies to identify the various surface phosphoproteins were done with specific antisera against surface located receptor and cell adhesion molecules.

Extracellular substrate phosphorylation catalyzed by ecto-kinase of intact cells or its related soluble form was studied in the blood system. Phosphorylation by ecto-kinases of a 135 kDa polypeptide in bovine serum having a 300 kDa precursor in plasma was previously observed (Cell.Biology of Ectoenzymes, Springer Verlag 1986): These substrates appear also to be phosphorylated selectively by the blood-derived PK activity. Remarkably in human plasma, a protein which appears to be a component of fibrinogen has been phosphorylated by the ecto-kinase as well as by the related plasma-derived PK making them prime candidates to catalyze in vivo phosphorylation.

403 CASEIN KINASE I: A Ca^{2+} ACTIVATED ENZYME?

Kaser, M.R., Cumming, D.V.E. and Ord, M.G. Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, U.K.

Casein kinase I is a prominent contributor to protein kinase activity in liver and thymus nuclei. The enzyme from both sources is selectively inhibited by Be^{2+}. After partial hepatectomy liver nuclear casein kinase I activity is raised at 5, 22 and 29 hours after operation. These peaks in activity are diminished by prior phentolamine administration which blocks the intracellular redistribution of Ca^{2+}. Autoradiography with liver nuclei has shown proteins of 118 kDa, the lamins, HMG14 and the slow components of both the 30 kDa doublet and histone H1 to have Be^{2+} sensitive phosphorylation. That of the latter two proteins is diminished if partially-hepatectomised rats have received phentolamine or propranolol. In thymus nuclei the lamins and the 30 kDa protein again show Be^{2+} sensitive phosphorylation as do proteins of 38, 36 and 25 kDa. Because of the blocking effects of phentolamine on nuclear protein kinase activity in regenerating liver, enzyme preparations from liver and thymus nuclei are being examined for possible Ca^{2+} activation of casein kinase I.

404 *PROTEIN PHOSPHORYLATION AND MEIOTIC CELL DIVISION IN XENOPUS OOCYTE.*

MULNER-LORILLON, O., BELLE R., HERMANN, J. and OZON, R. *Laboratoire de Physiologie de la Reproduction (UA CNRS, 555) Université Pierre et Marie Curie, 4 Place Jussieu, 75252 Paris Cédex 05, France.*

Progesterone induces the first meiotic cell division (meiotic maturation) of Xenopus full-grown oocytes by a mechanism involving protein phosphorylation/dephosphorylation. The hormone provokes a decrease in the level of phosphorylation of a protein (Mp), substrate of both cAMP-dependent protein kinase and protein phosphatase-1. In its dephosphorylated form, Mp initiates the formation of a cytoplasmic transferrable maturation factor (MPF) which activity is associated with a burst in cAMP-independent phosphorylation.

- Among the numerous proteins which are phosphorylated at the time of MPF appearance, we have characterized one protein of 47 KDa. Our results show that :

- *the p47 is phosphorylated at threonine residues ;*
- *it is detectable in the cytoplasm of enucleated oocytes induced to mature by MPF transfer ;*
- *it appears as early as 15 min. after MPF microinjection ;*
- *as already shown (HERMANN et al, Proc. Natl. Acad. Sci. U.S. 81, 5150, 1984) phosphatase inhibitors (2-glycerophosphate) facilated progesterone-induced maturation. The phosphorylation of p47 takes place earlier in 2-glycerophosphate-treated oocyte than in control oocyte.*

- We have purified from Xenopus oocytes a caseinkinase II-like activity on the following criteria :

- *it phosphorylates casein at threonine and serine residues ;*
- *it utilizes both ATP and GTP as phosphoryl donors ;*
- *it is inhibited by heparin.*

This casein kinase II-like activity isolated from the oocyte is capable to phosphorylate in vitro at threonine residues, a p47 preparation.

405 CYCLIC AMP ALTERS THE DISTRIBUTION OF CYCLIC AMP-DEPENDENT PROTEIN KINASE ON MICROTUBULES OF DROSOPHILA MELANOGASTER

Géza Ádám and Peter Friedrich. Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, H-1502, Budapest, P.O. Box 7, Hungary

Mammalian microtubules have been shown to bind cAMP-dependent protein kinase /PK-A/ via microtubule associated protein 2 /MAP-2/ /cf. Vallee, Bloom, Theurkauf, J.Cell Biol. 99, 38s /1984//. Bound PK-A is probably involved in phosphorylation of MAP-2, which in turn would influence microtubular dynamics.

We prepared microtubules by polymerization with taxol in head supernatants of wild type /Canton-S/ Drosophila. The phosphorylation of microtubular proteins by endogenous protein kinases has been characterized. cAMP markedly increased the incorporation of ^{32}P into several proteins, with the notable exception of tubulin, as revealed by SDS-gelelectrophoresis followed by autoradiography. Accordingly, the fruit fly microtubular protein contained PK-A, which was quantified enzymatically with pig brain MAP-2 as substrate and with the specific protein /Walsh/ inhibitor. The microtubular protein contained mainly PK-A holoenzyme and some catalytic subunit. Addition of cAMP drastically changed this picture: bound holoenzyme fell almost to zero, whereas bound C markedly increased. This cAMP treatment also induced the dephosphorylation of regulatory subunit of PK-A. The data conform to the idea that in the dunce memory--mutant of Drosophila, which has permanently high cAMP level, the distribution of PK-A in nerve cells differs from normal.

406 CYCLIC AMP-DEPENDENT PROTEIN PHOSPHORYLATION IN LARVAL BRAINS OF DROSOPHILA
Piroska Dévay, Ildikó Láng and Peter Friedrich. Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Budapest, P.O. Box 7, H-1502 Hungary

Earlier experiments with fly head homogenates /Dévay, Pintér, Yalcin, Friedrich: Neuroscience, in press/ suggested that in the $dunce^{M11}$ memory-mutant of Drosophila melanogaster the regualtory subunit of cAMP-dependent protein kinase /PK-A/ is shifted toward the dephosphorylated form as compared with the wild type, which may affect the substrate specificity in vivo of PK-A.

We made an attempt to characterize substrate proteins of PK-A in vivo. Surviving larvel brains were incubated in Ringer solution at 25°C in the presence of drugs that activated adenylate cyclase /e.g. octopamine and forskolin/. The consequential rise in cAMP level was accompanied by the labelling, from endogenous $[^{32}P]$ATP produced by preincubation of brains with $[^{32}P]$-orthophosphate, of a 27 kD protein /pI= 6.5/, as revealed by autoradiography of 2D-electrophoretic gels. Labelling of this protein was also affected by the K^+-channel blocker 4-aminopyridine.
This compound when fed to flies changed their learning index in behavioural tests. These findings suggest that the 27 kD substrate of PK-A is related to a K^+-channel. A putative cellular-molecular model incorporating most pertinent data on fruit flies, as well as the mechanism of classical conditioning described by others for the marine mollusks Aplysia and Hermissenda, is presented for the associative learning of Drosophila.

407 PROTEIN PHOSPHORYLATION IN PURE CHOLINERGIC SYNAPTOSOMES
X.Guitart, J.Blasi, C.Solsona and J.Marsal. Department of Histology and Cell Biology. Faculty of Medicine. University of Barcelona. Hospital de Bellvitge.Barcelona. Spain.

Protein phosphorylation has been related to the coupling of stimulus-secretion in different cellular systems. Using a pure cholinergic synaptosomal preparation obtained from the electric organ of Torpedo marmorata, we have measured the protein phosphorylation occurring within the synaptosomes related to acetylcholine release. Using depolarizing agents as an increase of extracellular potassium concentration, veratridine or gramicidin or by adding the calcium ionophore A-23187 we observed an increase in the incorporation of phosphorus into several proteins. This protein phosphorylation has been shown to depend on the presence of calcium in the medium of reaction. We have also tested the effect of tetrodotoxin that showed a reversing effect on veratridine induced protein phosphosphorylation in several bands.Botulinum toxin, that inhibits acetylcholine release also inhibits protein phosphorylation of several bands. We conclude that phosphorylation state of some proteins are affected by depolarization and these changes can be related to acetylcholine release.

408 EFFECT OF THYROXINE ON THE Ca^{2+} ATPase AND PROTEIN PHOSPHORYLATION IN HEART AND SKELETAL MUSCLES CELL MEMBRANES OF RABBIT.
Konrad S. Famulski, Grażyna Szymańska, Antoni Wrzosek and M. Gabriela Sarzała, Nencki Institute of Experimental Biology, The Polish Academy of Sciences, Warsaw 02-093, Pasteura 3, Poland.

Thyroid hormones affect the metabolism and the mechanical properties of heart and skeletal muscles. It is known that thyroxine stimulates the synthesis of Na^+/K^+ATPase, a sarcolemmal enzyme responsible for the repolarisation phase of the contracting heart and muscle cell. Sarcolemma from these cells posses two other distinct features. They are calmodulin-dependent Ca^{2+}ATPase and endogenous cAMP-dependent protein phosphorylation. The latter system activates the Ca^{2+}ATPase and the calcium transport across these membranes. Both systems are likely involved in the regulation of the relaxation rate of the cell. The properties of heart and skeletal muscles cell membranes isolated from hyperthyroid animals differ as compared to the control preparations. The activity of calmodulin-dependent Ca^{2+}ATPase is higher in membranes from hormone-treated animals. The kinetic evaluation of the Ca^{2+}ATPase activity revealed that the V_{max} value of the enzyme is altered. The cAMP-dependent protein phosphorylation, on the other hand is markedly inhibited. In membranes from skeletal muscles the inhibition of protein phosphorylation concerns the phosphate incorporation into peptides of app. molecular masses ranging from 25 kDa to 39 kDa. In heart membranes notably the phosphorylation of phospholamban is affected. The possible mechanism explaining parallel activation of Ca^{2+}ATPase and the protein phosphorylation inhibition will be proposed.

409 OSCILLATORY STATES AND COVALENT MODIFICATIONS OF L-ASPARAGINASE BY PHOSPHORYLATION/DEPHOSPHORYLATION. Stephan Jerebzoff, Simonne Jerebzoff-Quintin. Plant Physiology Center, C.N.R.S. (U.A. 241), Toulouse University, 118 route de Narbonne, 31062 Toulouse Cedex, France.

Rhythmic activity of L-asparaginase from *Leptosphaeria michotii* was dependent on a reversible phosphorylation process ; a protein complex involving asparaginase, protein kinase and a protein phosphatase was isolated and identified (1,2).

Phosphorylation of asparaginase complex by $[\gamma^{32}P]ATP\text{-}Mg^{++}$ underwent in alkaline and acid conditions ; it was not due to the incorporation of the nucleotide, as indicated by $[\alpha^{32}P]ATP\text{-}Mg^{++}$ experiments. Kinetics of phosphorylation and also dephosphorylation of the protein complex were reported. The Mg^{++}-dependent protein phosphatase (M_r 60 kDa) could be stimulated by Zn^{++} ; it was active at pH 8.6 but not at pH 6.0. The properties of this enzyme were analyzed.

In vivo incorporation of ^{32}Pi at different phases of the rhythm of asparaginase activity agreed with *in vitro* experiments.

(1) Jerebzoff S. and Jerebzoff-Quintin S. FEBS Lett., 1984, 171, 67-71.
(2) Jerebzoff-Quintin S. and Jerebzoff S. Physiol. Plant., 1985, 64, 74-80.

410 IMMUNOLOGICAL CHARACTERIZATION AND LOCALIZATION OF PHOSPHOLAMBAN IN CARDIAC MUSCLE. W. Schulze, M. Holtzhauer. Central Institute of Cardiovascular Research. Acad. Sciences of the GDR, 1115 Berlin, GDR

Phospholamban, a 22,000-Dalton proteolipid first described by Tada et al. (J. Biol. Chem. 249, 6174, 1974), can regulate the Ca-transport system in cardiac muscle by cAMP-depending phosphorylation. However the question concerning structure and function of phospholamban remains unsolved. Although cardiac sarcoplasmic reticulum (SR) might be the main site at which phospholamban exerts its action, an alternative localization is likewise possible. To solve this problem immuno-gold technique has been used in rat rabbit and pig hearts. Antisera against phospholamban were produced in rabbits (Holtzhauer et al. Biomed. Biochim. Acta, in press). Electroblot analysis demonstrates specific binding of the antisera to purified phospholamban and to phospholamban in preparations of cardiac SR and sarcolemma (SL). Goat anti-rabbit immunoglobulin linked to 15 nm gold particles were prepared and their reaction was tested with phospholamban antisera on nitrocellulose sheets. In preliminary experiments using pre- and postembedding techniques with Lowicryl K4M ultrathin sections, antigenic domains against phospholamban antisera were localized. Gold particles are found over nonjunctional SR and in subsarcolemmal vesicles. These vesicles are labeled mainly in preinbedded preparations. Therefore, penetration artefacts can not be excluded. Whether the vesicles originate from SR or SL remains to be elucidated. Minimal nonspecific background staining of all muscular structures were seen with preincubated normal rabbit serum. Further studies are in progress.

411 Ba^{2+} ION INTERACTIONS WITH Ca^{2+} TRANSPORT SYSTEMS OF MITOCHONDRIA
L.G. Lukács and A. Fonyó, Department of Physiology, Semmelweis Medical University, Budapest, H-1444 POB 259 Hungary

The effect of Ba^{2+} ions on Ca^{2+} movements (uptake and release) were measured in liver and in heart mitochondria. Under the conditions employed Ba^{2+} did not affect Ca^{2+} uptake neither in liver nor in heart. In contrast to this, Ba^{2+} ions were strongly inhibitory on Ca^{2+} release in both types of mitochondria but with very different characteristics.

In heart mitochondria, where Ca^{2+} release is Na^{+}-dependent, 0.9 μM of added $BaCl_2$ inhibited the Ca^{2+} release by 50%. At the same time Ba^{2+} ions did not enter the matrix space. The inhibition by Ba^{2+} was non-competitive with respect to added Na^{+}. The Ca^{2+} release pathway has a regulatory Ca^{2+} binding site at the outer surface of the membrane: Ba ions bind to this site, as it was verified with double inhibitor titration.

In liver mitochondria where Ca^{2+} release is Na^{+}-independent, about 40 μM of $BaCl_2$ was needed to inhibit Ca^{2+} release by 50%. Ba^{2+} was taken up slowly into these mitochondria: inhibition of Ba^{2+} uptake by ruthenium red prevented the inhibitory action of the former. Uptake of Ba^{2+} and inhibition of Ca^{2+} release run in parallel. Thus Ba^{2+} ions are inhibitory only after being taken up into the matrix. As the Ba^{2+} inhi bition of the Ca^{2+} release was competitive with respect to the intra-mitochondrial calcium, it is proposed that Ba^{2+} ions are bound to the internal substrate binding site of the release pathway.

The Ca^{2+} release system of heart and liver thus can be distinguished not only on the basis of their Na^{+}-sensitivity but also by their divalent cation binding sites.

412 IMMUNOCYTOCHEMICAL INVESTIGATION OF NATIVE MATRIX GRANULES IN THE HEART MITOCHONDRION.
R.C.Hertsens, Inge Bernaert, M.Joniau°, W.A.Jacob. University of Antwerp, UIA, B-2610 Antwerp (Wilrijk), Belgium. (°) I.R.C., K.U.Leuven, Campus Kortrijk, B-8500 Kortrijk, Belgium.

Since Palade (1952) and Sjöstrand (1953) first described mitochondrial matrix granules, many investigations were done to find the composition and the physiological significance of these structures. There is a general agreement that native matrix granules (NMG) are spherical osmiophilic inclusions which are located within the space, bound by the inner membrane. Sometimes, they are located very close to the cristae mitochondriales and a fusion cannot always be excluded. The diameter of these NMG's is about 300 Å but depends on the type of mitochondrion and its physiological state.

The NMG's are randomly dispersed in the matrix under normal conditions. Moderate stimulation with catecholamines shifts the NMG's to the periphery of the mitochondrion. After heavy stimulation no NMG's are observed in the matrix (Jacob, et al 1972). It is believed that the content of these granules is incorporated in the inner mitochondrial membrane (Jacob and Hertsens, 1984) or transported outside the mitochondrion. After a long periode of rest, the NMG's reappear in the matrix. The hypothesis that these NMG's are pools of constituents of the inner membrane is tackled with post embedded immunocytochemistry.

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413 ADAPTIVE RESPONSES TO COLD OF PEROXISOMES AND MITOCHONDRIA IN RAT BROWN ADIPOSE TISSUE. Stefan Alexson (1) and Tahashi Hashimoto (2). (1) The Wenner-Gren Institute. University of Stockholm, S-106 91 Stockholm, Sweden, and (2) Shinshu University School of Medicine, Matsumoto, Nagano 390, Japan

Brown adipose tissue is the main site for nonshivering thermogenesis. The heat production is mainly due to an uncoupled oxidation of fatty acids. For this purpose brown adipose tissue is well equipped, having a rich vascularisation, rich sympathetic innervation, high content of mitochondria which contain thermogenin (the brown-adipose-tissue specific uncoupling protein), and high concentrations of respiratory enzymes and fatty acid metabolising enzymes (Cannon and Nedergaard, 1985). Besides the mitochondria, brown adipose tissue also contains peroxisomes able to oxidize fatty acids (Nedergaard et al., 1980; Alexson and Cannon, 1984). In the present work we have studied changes in mitochondrial and peroxisomal enzymes during acclimation to cold by following changes in enzyme activities, immunoprecipitable protein and immunoprecipitable enzyme activity. The enzyme extract was prepared by centrifugation of a homogenate (Ultra Turrax) at 100 000 x g for 30 min. The enzyme activities were measured in the supernatant. Some peroxisomal enzymes increased several fold after a lag phase of about 3 days (catalase and acyl-CoA oxidase), whereas other peroxisomal enzymes appeared more or less unchanged (bifunctional enzyme and thiolase). Mitochondrial β-oxidation enzymes were only slightly increased or unchanged (per g ww) in cold-acclimated animals compared to control. However, the activity (per g ww) of the mitochondrial enzymes appeared to be transiently decreased during the first week in the cold. This decrease appeared to be well correlated to a decrease in in the amount of immunoprecipitable protein. Lactate dehydrogenase activity was followed as an example of a cytosolic enzyme. The activity was doubled after 3 days in the cold, but in cold-acclimated animals it was about the same as in the control state. Thus, all enzymes in each compartment (mitochondria, peroxisomes or cytosol) showed a similar response to cold, whereas each compartment appeared to respond as a unit.

414 PHOSPHORYLATION OF THE TWO LIGHT-HARVESTING CHLOROPHYLL a/b BINDING PROTEIN COMPLEX (LHC-II) POLYPEPTIDES AND THE ATP-INDUCED PHOTOSYSTEM II FLUORESCENCE DECLINE. Khalid Islam, Centro CNR, Dipartimento di Biologia, Universita di Milano, Via Celoria 26, 20133 Milano, Italy.

The phosphorylation of the LHC-II polypeptides has been proposed to regulate the distribution of absorbed excitation energy between the two photosystems, PSI & PSII (Allen et al 1981, NATURE 291, 25) and to lead to a decline in the PSII room temperature fluorescence emission (Fm). However, we have recently shown there is little correlation between the level of phosphorylation of the LHC-II polypeptides and the Fm decline in the presence of 5 mM Mg^{2+} (Islam & Jennings 1985, BBA 810, 518). The relationship between these two phenomena has been further examined using different preparations of isolated spinach chloroplasts. The stoichiometries of phosphorylation of the 24kda and 25kda LHC-II polypeptides has been determined by quantitative SDS-PAGE. The results show that (a) the relative stoichiometry of 24kda : 25kda polypeptide was 1 : 3 in isolated chloroplasts and purified LHC-II complexes (b) the initial rate of phosphorylation of the 24kda polypeptide exceeded that of the 25kda about 3x although the final extents were similar (c) while the extent of phosphorylation varied, 0.15-1.0 mole PO_4/mole LHC-II polypeptide, the Fm decline was ca. 15-20% and (d) the initial rate, determined in the first 2 min period, also varied by upto 8-fold but the half-time for the Fm decline remained ca. 2-3 min. These results also suggest that in the presence of 5 mM Mg^{2+} there is little or no direct correlation between either the extent or the initial rate of phosphorylation of the LHC-II polypeptides and observed Fm decline.

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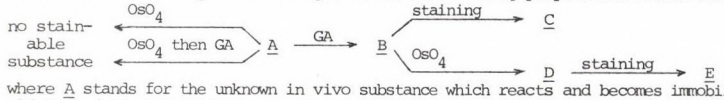
415 IN SITU INVESTIGATIONS ON THE INTRATHYLAKOIDAL DENSE SUBSTANCE OF CHLOROPLASTS. A.Keresztes, Department of Plant Anatomy, Eötvös Loránd University, H-1088 Budapest,Muzeum krt.4/A, Hungary

In chloroplasts from several different sources the lumina of thylakoids are electron dense while the thylakoid membranes are hardly stainable after the conventional preparation. In order to characterize the dense substance we carried out fixation and enzymic digestion probes on sunflower /Helianthus annuus L./ cotyledons. Changes in the lumen during preparation can be summarized as follows:



where A stands for the unknown in vivo substance which reacts and becomes immobilized with glutaraldehyde /GA/, but diffuses out of the thylakoid upon the effect of OsO_4. B can be solved out of the sections by distilled water, so it can not be of lipidic nature. After having reverted it by deosmification from D it is insensitive to proteolytic enzymes, so it may not be of proteinaceous nature. B is not electron dense in itself, but reduces heavy metal ions forming C, the dense substance proper which can not be distinguished from E on the basis of the densities. Direct investigations on the nature of A substance are under way.

416 CHLOROPLAST AND PROTOPLAST DEVELOPMENT IN FLUORDEOXYURIDINE(FUDR) TREATED CELLS OF UNICELLULAR GREEN ALGA SCENEDESMUS QUADRICAUDA. Tomáš Kalina (1), Vilém Zachleder (2). (1) Dept. Cryptogam. Botany, Faculty of science, Charles Univ., Benátská 2, Praha 2, 12800 and (2) Microbiol. Inst. ČSAV, Opatovický mlýn, Třeboň 37981, Czechoslovakia

25 ug/ml FUDR (DNA synthesis & replication inhibitor) was addet to the synchronous culture (clt) at 4th or 6th hour of the life cycle. The clts were grown in the dark and studied 2 times : first when the controle clt were just in division and second 10 hrs latter. FUDR prolongates the period of the life cycle for the treated clt, it does not divide in the time when the cells of the control do so. In the treated clt studied 10 hrs latter 2 subpopulations of cells occur : the first one is formed by cells which not divide being influenced by FUDR earlier then reproduction sequences could be induced. The second subpopulation consists of sporangia containing 8 daughter protoplasts of unequal size from which only one contains the nucleus. Each protoplast forms its own cell wall. Chloroplast development is similar as in controle clt, nucleoids not replicate.

417 BIOGENESIS OF PEROXISOMES: IMMUNOELECTRON MICROSCOPIC STUDIES IN REGENERATING RAT LIVER.
Kouta Yamamoto, Alfred Völkl and H.Dariush Fahimi, Department of Anatomy, II. Division, University of Heidelberg, D-6900 Heidelberg, Fed.Rep.Germany.

The regeneration of rat liver after partial hepatectomy (PH) is an excellent model for the study of biogenesis of mammalian peroxisomes. We reported recently our observations on cytochemical staining of peroxisomes for catalase and uricase in regenerating rat hepatocytes (K.Yamamoto et al., 1985; Arbeitstagung der Anatom.Gesellschaft in Würzburg). In the present study we have used the technique of immunoelectron microscopy to trace the intracellular pathway of peroxisomal proteins.

Female Wistar rats were subjected to PH and livers were fixed by vascular perfusion with glutaraldehyde at various time intervals after the operation and embedded in Epon and Lowicryl K4M. Monospecific antisera against catalase and acyl-CoA oxidase were raised in rabbits and their specificities were assessed by immunodiffusion and immunoblotting. Ultrathin sections were incubated with antisera followed by labelling with protein A-gold and examination in an electron microscope.

The peroxisomes in regenerating rat liver showed several distinctive morphological features: a) marked variation in shape and size and b) evidence of cleavage or budding. By immunoelectron microscopy, all peroxisomes, irrespective of size and configuration were strongly positive for catalase showing numerous gold particles distributed over their matrix. The peroxisomal cores were usually free or contained a few gold particles. Sections incubated with the antibody against acyl-CoA oxidase showed essentially a similar distribution of gold particles, although the labelling density was weaker than with catalase. Other cell organelles were consistently negative, as were the control sections. These observations demonstrate that in regenerating rat liver peroxisomes undergo remarkable changes in shape, size and distribution suggesting their de novo formation by fission and fusion. The localization of catalase and acyl-CoA oxidase in all peroxisomes (presumably new and old) is consistent with the concept of transfer of matrix proteins from preexisting particles to new ones. Supported by DFG (Fa 146/1-3).

418 MICROTUBULE-ASSOCIATED PROTEINS OF HIGH M_r: FACTS AND PERSPECTIVES.
Gerhard Wiche, Christiane Koszka, Roland Foisner, Harald Herrmann and Reinhard Furtner, Institute of Biochemistry, University of Vienna, A-1090 Vienna, Austria.

High M_r polypeptides, together with tubulin, are the major constituents of microtubules assembled from mammalian brain. Originally only two components, MAP-1 (M_r = 350,000) and MAP-2 (M_r = 300,000) were distinguished. It turned out, however, that both MAP components split up into a number of bands upon high resolution gel electrophoresis. MAP-1 and MAP-2 both stimulate the assembly of microtubules in vitro and form long protrusions on the polymer's surface. Therefore, they are considered as strong candidates not only for regulators of microtubule assembly, but also for mediators of microtubule interactions. The issue whether MAP-1 and MAP-2 in general are constituents of microtubules including those of nonneuronal origin remained controversial for some time. Recent evidence, however, points towards a widespread occurrence of both MAPs including nonneuronal cells[1].

The widespread occurrence and structural diversity of MAPs could be important for the proposed function of MAPs as crosslinkers between microtubules and their various interaction partners. In fact, if the binding of MAPs to different interaction partners is specific and inherent to the structure of MAPs, they could be vital elements in determining the structure and shape of the cytoplasmic space. Recently our work has been focused on testing this hypothesis by isolating and characterizing MAPs from sources other than brain and structurally analysing MAP subcomponents. The isolation of MAP-1 and MAP-2 from cultured cells by repeated rounds of in vitro polymerization and depolymerization was found ineffective, largly because of proteolytic breakdown and partial retention of both MAPs in the insoluble fractions. Microtubules polymerized from isotonic cell extracts of cultured cells by taxol, however, were found to be enriched in both MAPs. Upon electrophoresis of taxol-polymerized microtubule preparations from glioma C6 cells on low percentage polyacrylamide gels, multiple protein bands in the M_r region of MAPs became apparent. The bands migrating in the MAP-1 and MAP-2 regions were immunoreactive with the respective antibodies to neuronal MAPs. The isolation of undegraded high M_r polypeptides, particularly MAP-2, from cultured cells was to a large extend dependent on the removal of free Ca-ions. Polypeptides immunologically and chemically related to neuronal high M_r MAPs were also isolated from rat liver.

The analysis of MAP subcomponents from mammalian brain recently led to the isolation of a novel high M_r microtubule binding protein. This polypeptide, although similar to MAP-2A in M_r, is clearly structurally different from MAP subcomponents, as revealed by peptide mapping and immunoblotting experiments. Immunological cross-reactivity was observed, however, with an antiserum raised against dynein from Tetrahymena. Partially purified preparations of this polypeptide exhibited ATPase activity which was suppressed by erythro-9(3-(2-hydroxynonyl))adenine (EHNA). In a limited survey of various cells and tissues, a protein of similar properties was positively identified in cultured rat glioma C6 cells and rat liver tissue[2]. It seems, therefore, that this newly identified high M_r protein, like MAPs, is not restricted to neuronal microtubules. The identification of this protein opens new perspectives regarding the functions of high M_r microtubule binding proteins.

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419 PURIFIED THYROID LYSOSOMES BIND TO IN VITRO RECONSTITUTED MICROTUBULES.

Gilles Mithieux, Françoise Bernier-Valentin and Bernard Rousset
INSERM U.197, Faculté de Médecine Alexis Carrel. 69372 Lyon. France

Numerous pharmacological and morphological studies suggest that microtubules play a role in intracellular transport of secretory vesicles. The postulated mechanism would involve the association of secretory granules to microtubules. In an attempt to define the molecular basis of such an interaction we have tried to reconstitute in vitro the association of a type of secretory vesicles (purified thyroid lysosomes containing the thyroid hormone precursor protein : thyroglobulin) to microtubules.

Microtubule proteins were purified from pig brain by the polymerization/depolymerization procedure and labeled with 125 I-Bolton Hunter reagent. Highly purified lysosomes (50 fold) were obtained from pig thyroid by isopycnic centrifugation on Percoll gradients. Microtubules were polymerized for 30 min at 37°C and incubated in the presence of lysosomes. The formation of microtubule-lysosome complexes was followed : 1) by electron microscopy (negative staining); 2) by a sedimentation method: incubation mixtures were layered on 0.75 M sucrose cushion and centrifuged at 10,000g for 5 min (under these conditions, lysosomes and microtubule-lysosome complexes sedimented whereas microtubules did not). The amount of microtubules bound to lysosomes was determined by measurement of the radioactivity of the pellets. The association phenomenon was time- and temperature-dependent ; at 25°C the formation of complexes began after a 5-10 min lag-period and reached a plateau between 25 and 35 min, the equilibrium was reached about twice more rapidly at 37°C. The amount of complexes formed after 30 min was dependent on the concentration of both lysosomes and microtubules. Lysosomes in close contact with microtubules were observed in both unfractionated lysosomes-microtubules mixtures and 10,000g pellets.

In conclusion, we have defined an acellular system in which the association of lysosomes to microtubules takes place. This experimental model shoud allow a) to identify the molecular species responsible for the interaction and b) to search for possible regulatory mechanism(s).

420 A MICROTUBULE-ASSOCIATED ATP-BINDING PROTEIN OF 180 KD IS PRESENT IN MAMMALIAN MITOTIC SPINDLES.

Karl-Heinz Doenges, Tore Kempf, Herwig Ponstingl, Institute of Cell- and Tumor Biology, German Cancer Research Center, D-6900 Heidelberg.

Microtubule proteins were purified from mouse Ehrlich ascites tumor cells by two cycles of polymerization in 0.1M PIPES, 1mM EGTA, 1mM $MgCl_2$, 0.1 % 2-mercaptoethanol, pH 6.9. Microtubules were assembled by incubating the C_2S solution at 37°C in the presence of 1mM GTP, addition of 2mM ATP resulted in a gradually contracting gel, as observed by Weisenberg & Cianci (J. Cell Biol. 99, 1527-1533, 1984) with material from brain. Gel electrophoresis of unpolymerized C_2S revealed two polypeptides of 190 and 180 kd as major constituents in addition to tubulin. A photoreactive analogue of ATP, γ^{32}P-8-azido-ATP, was mixed with C_2S for 3 min and the mixture was irradiated at 254 nm for 2 min. In the irradiated sample both high molecular weight proteins incorporated the label covalently. The polypeptides were purified by preparative gel electrophoresis and used to raise antibodies in rabbits. In indirect immunofluorescence studies with PtK2 and HeLa cells the antiserum to the 180 kd component stained mitotic spindles and poles. In interphase, a variable number of coarse bright speckles appeared in an otherwise weakly stained nucleus (supported by the Deutsche Forschungsgemeinschaft).

421 SUBCELLULAR MOTILITY INVESTIGATED WITH NANOVID MICROSCOPY. M. De Brabander[1], R. Nuydens[1], G. Geuens[1], H. Geerts[1], C. Hopkins[2]. (1) Division of Cellular Biology and Chemotherapy, Department of Life Sciences, Janssen Pharmaceutica, B-2340 Beerse, Belgium and (2) Department of Medical Cell Biology, Medical School, University of Liverpool, Liverpool L69 3BX, England

Within vertebrate cells many membrane lined organelles migrate along microtubules in a saltatory and energy dependent way. The mechanism involved, the specificity and the functional consequences are still poorly understood. In order to be able to follow the motility of defined subcellular components we have developed a new approach called Nanometre Particle Video (Nanovid) microscopy. The method uses colloidal gold particles of submicroscopic size (10-40 nm diameter) as non-specific or specific (through antibodies or other ligands) markers. The gold particles are not visible to the eye in the light microscope. However by using the contrast enhancement and image improvement capacity of a video microscopy system (Hamamatsu C-1966) individual gold particles can easily be detected and discerned from cell organelles with transmitted or incident bright field illumination.

Microinjected gold particles coated with albumin and stabilized with polyethylene glycol bearing a negative surface charge are transported in an energy dependent saltatory way along microtubules at the same velocity and frequency as endogenous organelles. Gold particles preincubated with cetylpyridine, having a positive charge, show predominantly Brownian motion. The transport mechanism is thus not specific for organelles. It may involve an ATP-ase that can interact with any particle of suitable charge (Adams, R.J. and Bray, J.D., Nature, 303, 718-720, 1983; Beckerle, M.C., J. Cell Biol. 98, 2126-2132, 1984).

To follow the binding, internalization and intracellular traffic of specific cell surface binding sites we incubated A431 cells with gold particles coupled to monoclonal antibodies to the transferrin binding site (ATR-gold, Hopkins, C.R., and Trowbridge, I.S., J. Cell Biol., 97, 508-521, 1983). The ATR-gold bound specifically to the peripheral free lamella. Initially, many particles performed two

dimensional Brownian motion on the cell surface in this area. Within few minutes they became trapped in a rim separating the lamella from the central cytoplasm. Here particles were endocytosed predominantly in small (± 50-100 nm) but also in large vesicles and cisternae. The endosomes became rapidly engaged in typical saltatory motion which was ATP and microtubule dependent. The direction of individual saltations (either centripetal or centrifugal) was unpredictable. Gold containing vesicles often showed several inward and outward migrations in succession. At no stage could a consistent uniform centripetal movement of endosomes be detected. Nevertheless, already after ± 15 min gold containing vesicles accumulated in the perinuclear region surrounding the centrosome. In this area as well as elsewhere in the cytoplasm, endosomes were often seen to fuse forming larger vesicles. However, fission of small vesicles from large ones and migration to the cell periphery was also frequently observed. With time, the accumulation in the centrosomal area continued until most of the gold was concentrated there after 4-6 hrs.
This process, unlike binding and endocytosis, was entirely arrested by nocodazole which disassembles microtubules and by taxol which replaces the radiating microtubule complex by a disorganized microtubule network.
Our data show that endosomes do not migrate unidirectionally towards the cell centre. They suggest that the bidirectional saltatory motion along microtubules is converted into a concentrative transport mechanism by trapping in the centrosomal area. The radial organization of the microtubule system may be the major determinant of the trapping mechanism. Statistically, the chance of multiple interactions of vesicles with different microtubules increases towards the focal point where the microtubule density is highest. Because each individual interaction may produce either a centrifugal or a centripetal force multiple interactions increase the probability of no net movement occuring. Moreover any centripetal movement leads to the formation of additional bonds while any centrifugal movement involves the breakage of bonds.

422 STRUCTURAL DYNAMICS OF MICROTUBULES IN VITRO MEDIATED BY ASSOCIATED PROTEINS

E. Unger, W. Vater, H. Müller, K.J. Böhm, Academy of Sciences of the GDR, Central Institute of Microbiology and Experimental Therapy, Department for Experimental Cell Research, DDR-6900 Jena, POB 73, GDR

In the presence of cofactors (e.g. Mg^{2+}, GTP) microtubule protein (MTP) assembles into microtubules (MTs). MTs are composed of protofilaments (pfs) the number of which (as average of the population) is characteristically related to the assembly conditions realized and especially to the content of microtubule-associated proteins (MAPs). During assembly increasing MAP activity enlarges the pf number and vice versa. Mean values were found about 12 for MAP-free pig brain tubulin and 14 for complete MTP with extremes of 8 and 16. Disturbances of assembly steady state (always monitored by turbidity) by changing MAP activity cause variations of the pf number, too. Consequently, the mean pf number of MT populations may be regarded as a dynamic property. Moreover, the pf number can probably be changed even in an individual MT.

Intermediate structures of MT formation are sheet-like assemblies with C-shaped profiles (C-sheets) that increase their pf number during the assembly course and are transformed into MTs by closing the tubule wall in dependence on the conditions realized. The subunit concentration should be of special importance to reach the pf number sufficient for the sheet conversion. Under some conditions (e.g. > 2 M glycerol, > 10 μM taxol) C-sheets coexist beside MTs for a certain time at steady state. These C-sheets disassemble slowly with an invariant pf number. Subunit addition to MT ends is obviously favoured to a lateral addition in form of pfs. We conclude that there should be a kind of critical concentration of MTP for formation of additional protofilaments in "open" assemblies that is higher than critical concentration for pf elongation. A lack of MAPs causes a percentual increase of sheets persistent. Disassembly of steady state C-sheets in favour of MT elongation, however, was not observed in this case.

The presence of histones in assembly processes (to enhance MAP effects) causes the formation of typical double-walled MTs (dwMTs) with an increased pf number in the inner MT that is surrounded by a wall of pf rings or spirals. The two tubulin layers are spaced by histones. Often, MTs are clustered with histones as embedding material. C-sheets are only one-sided covered by histones. MTs with more than two concentric walls have never been found. It was derived that the binding sites for histones are located only on those regions of pfs that form the MT surface, expressing a second axis of polarity within pfs. dwMT are realized by "face-to-face" orientation of pfs. Alternating pf orientation in flat Zn-sheets causes, consequently, sheet heaps found with up to 5 aligned sheets. Astonishingly, some double Zn-sheets, formed by histone action, are curved at their edges or even change over into dwMT-like structures. The alternating pf polarity of Zn-sheets had to be interrupted in these curved areas.

It can be shown that structural properties of MTP assemblies are convertible by MAPs and some other analogously acting proteins able to bind at the MT surface. The structure of assemblies and their dynamics are markedly determined by tubulin-binding proteins; some are powerful structure mediators that may influence also the physiological relevance of different MTP assemblies.

423 PRESENCE OF ACTIN AND MYOSIN IN ISOLATED CILIA FROM QUAIL OVIDUCT. B. Chailley, K. Bork, P. Gounon, D. Sandoz. Laboratoire de Biologie Cellulaire CNRS, 67 rue Maurice Günsbourg, 94200 Ivry sur Seine, France.

Previous immunocytochemical studies have revealed the presence of actin and myosin associated to axonemes of ciliated epithelium from quail oviduct(1). These results were obtained from isolated and demembranated ciliated cells. Demembranation using Triton X100 could induce protein redistribution and thus wrong localization could be suspected. In order to avoid this possible artefact, these two contractile proteins have been searched in cilia isolated with their membrane.

Cilia were isolated by mechanical stirring in a physiological saline solution. Cilia fraction purity was controled in phase contrast and electron microscopies. Cilium scission occurs in the ciliary necklace area as well as in the case of the hormonal-induced deciliation (2).

The presence of actin and myosin was detected on isolated cilia by indirect immunofluorescence, using purified monospecific antibodies against actin and myosin from frog striated muscle (3). The results were compared to those obtained with antitubulin antibody which stains the whole length of cilium : actin is localized in a third part, and myosin is detected at the cilium base. The actin presence was confirmed by immunoblotting of proteins from purified fractions of isolated cilia in one-and two dimensional PAGE gels. The 200 kDa band is recognize by antibody against myosin from striated muscle, while a monoclonal antibody recognizing a myosin of non muscular cell does not labelled the 200 kDa band of cilia. The myosin associated to axonemes is thus immunologically related to myosin from striated muscle.

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424 REGULATION OF ACTIN FILAMENT ASSEMBLY AND STRUCTURE.

A.G. Weeds, J. Gooch and B. Pope, MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, England.

Many of the motile events of cells including locomotion, morphological changes, phagocytosis and cytokinesis involve actin and its associated proteins. Changes in the localization of actin and its supramolecular organisation must occur rapidly in response to different stimuli. These changes involve the reversible interaction of a variety of actin binding proteins and control of the polymerization state of actin itself. One of the signals involved is the intracellular calcium ion concentration. It is therefore important to elucidate the mechanisms by which calcium and other intracellular signalling agents regulate cytoskeletal architecture and motility.

Actin-containing structures in cells include microvillar bundles, isotropic meshworks and stress fibres. Many of the proteins involved in the formation of these structures show no calcium-sensitivity: these include fimbrin and fascin in actin bundles and filamin or actin binding protein in isotropic gels. There also exist calcium-sensitive bundling proteins like alpha-actinin, but control of cytoskeletal structure may frequently involve restriction of actin filament length and changes in the monomer-polymer equilibrium. Proteins that cap the ends of actin filaments will affect this equilibrium and profilins, which bind G-actin, amplify this effect. A large number of these proteins also sever actin filaments. These include gelsolins and villin from vertebrates and fragmin and severin from amoeboid cells. Gelsolin was originally identified in macrophages [1], but is also found in platelets, muscle and many other tissues. In addition it is present at similar concentrations in blood plasma [2].

We have isolated gelsolin from pig plasma and shown its relationship to pig platelet gelsolin based on amino-terminal sequences. Its interaction with actin has been studied by electron microscopy, viscometry and using fluorescently labelled actin as a probe. Its properties include: 1) it severs actin filaments and caps the free barbed ends [3]; 2) this capping activity raises the critical concentration and thereby promotes depolymerization [4]; 3) it binds monomeric actin to form a binary complex in the absence of calcium and a ternary complex with two actin subunits in the presence of calcium [5]; 4) these complexes nucleate actin polymerization; 5) it binds two moles of calcium with Kd $\sim$ 1 uM; 6) micro-injection into cultured epithelial cells at about 1 nM intracellular concentration results in rapid destruction of stress fibres [6].

The calcium sensitivity of actin-gelsolin interactions appears to differ between different isoforms. Rabbit macrophage gelsolin requires calcium for its capping and severing activities [7] and human platelet gelsolin does not bind monomeric actin without calcium [8]. By contrast, pig plasma gelsolin binds actin both in the presence and absence of calcium. To explore these differences in calcium sensitivity further, we have used radio-iodinated gelsolin to monitor binding to F-actin. Results showed that in calcium over 90% of the gelsolin co-sediments with F-actin in a Beckman Airfuge, while in EGTA only about 50% of the counts are pelleted. The remainder is in the form of a binary complex with G-actin. These observations are independent of actin or gelsolin concentrations upto the point where actin filaments are so short that they do not sediment. Using actin-Sepharose affinity columns, two

forms of gelsolin have been separated. One does not bind to F-actin or sever filaments in EGTA and is therefore fully calcium-sensitive, while the other binds equally well in the presence or absence of calcium. Both however, form complexes with monomeric actin. Thus the calcium sensitive gelsolin appears to discriminate between monomeric and polymeric conformations of actin.

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425 LOCALIZATION OF MICROFILAMENT BINDING PROTEINS BY IMMUNOCYTOCHEMISTRY.

Jan De Mey, Gabriele Langanger and Marc De Brabander, Laboratory of Biochemical Cytology, Department of Cellular Biology and Chemotherapy, Janssen Pharmaceutica Research Laboratories, B-2340 Beerse, Belgium.

The motile processes of non-muscle cells are to a large extent mediated by F-actin microfilaments and accessory proteins. These "actin-binding" proteins play important roles in structural aspects (e.g. bundling, interactions with membranes and other filament types) in the regulation of actin assembly-disassembly, and in the generation of mechanochemical phenomena.
Cultured monolayers are widely used as a model system for visualizing the intracellular distribution of actin-binding proteins. For a deeper understanding of the molecular arrangements of these proteins, it is essential to use high resolution ultrastructural localisation techniques.
An "optimal" method should comply with the following criteria :
1. Probes containing a punctate maker with high electron density and spatial resolution should be used. Such probes are compatible with contrasting techniques that give adequate ultrastructural delineation of preserved cell structures. Small (5-10 nm) gold probes, especially those containing directly labelled antibodies, are very suitable.
2. The distribution of the punctate maker must faithfully reflect the 3-D distribution of the antigenic sites. The marking must be quantitative along the linear cytoskeletal elements. It follows that during incubation with the immune reagents, the antigenic sites along filaments must be accessible and reactive. This criterium cannot be met by "post-embedding" on-grid methods.
3. The filamentous systems must be sectioned parallel to their main orientation. In cultured cells, this is close to and parallel with the growth substrate. Then only is it possible to correlate the distribution of the marker with well delineated structures visible in the electron microscope, as well as with data obtained from whole cells at the light microscopic level.
Light microscopic techniques for cultured cells generally use whole cells that are pre-fixed in situ and made permeable to the immune probes. An analoguous E.M. approach is called pre-embedding labelling. Here, fixation is often preceded by a brief lysis in detergent. This damages membrane systems, the cytoplasmic ground substance and accessory proteins that are very susceptible to extractions (e.g. filamin). In addition, the marker often penetrates poorly in the densely packed structures. Recent work from our group provides examples and has in spite of the disadvantages, given insight in the molecular architecture of stress-fibers, especially in the arrangement of myosin. Using antibodies with well defined myosin fragment specificity, it was concluded that myosin in stress-fibers is organized in the form of bipolar filaments located in between the α-actinin containing dense bodies.
There is however a need for further methodological improvements in order to eliminate the drawbacks of the pre-embedding approach. In a separate communication (Langanger et al.) we introduce an alternative method which involves embedding of monolayers in a reversibly polymerized acrylamide, freezing, and cutting ultrathin or semithin frozen sections, parallel to the substrate. Sections on grids are treated to depolymerize the polyacrylamide. After immunolabelling they are processed in various ways for EM or LM observations. This entirely aqueous approach looks very promising and preliminary results of light and electron microscopic localizations will be presented. Reversible embedding (immuno)cytochemistry, performed in this way could become a widely useful method entailing many of the requirements for an optimal localization technique.

426 ANALYSIS OF CYTOPLASMIC ACTOMYOSIN FUNCTIONS BY MICROINJECTION

Brigitte M. Jockusch, Annette Füchtbauer, Anke Huckriede and Bernd Höner
Developmental Biology Unit, University of Bielefeld, D-4800 Bielefeld 1, FRG

The cytoplasmic actomyosin system of eukaryotic cells is responsible for a variety of motile processes, such as intracellular organelle transport, locomotion, cytokinesis, maintenance of cell:cell and cell:substratum contacts. All these processes have in common that the key structural unit, the actin filament, must interact specifically with cellular membranes, either to create motility or to exert tension. Each of these processes is correlated with the formation of supramolecular aggregates in which actin filaments are held in place and are regulated in length by a variety of structural proteins (actin-binding proteins). While many of these proteins have been biochemically characterized (Ref.1,2), their functions as actomyosin modulators in the living cell are poorly understood so far. Microinjection into single cells of such proteins, or, of high affinity antibodies against them, offers a specific tool to interfere with cellular equilibria in the dynamic architecture of the actomyosin system. Using microinjection with glass capillaries and subsequent analysis of the injected cells by immunofluorescence, phase and reflection contrast microscopy as well as electron microscopy, we studied the effect of unbalancing intracellular equilibria of actin-binding proteins on the following: (1). The quirements of fibroblastic and epithelial tissue culture cells to express actin filament bundles (stress fibers); (2). The correlation between stress fiber expression, cellular morphology and cell:substratum attachment; (3). The ability of such cells to restore their previous stress fiber complement after microinjection-induced disintegration; (4). The stability of the microfilament organization in locomoting cells. We found specific differences in the functional role of different actin-binding proteins in these processes (Ref.3-6).

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427 ANALYSIS OF THE FUNCTIONS OF CYTOPLASMIC ACTOMYOSIN IN CELL-FREE MODELS.

Karl-Ernst Wohlfarth-Bottermann, Institute for Cytology, University of Bonn, Ulrich-Haberland-Straße 61a, D-5300 Bonn 1, FRG

Cell-free models should offer "in situ-conditions" to study the physiology of contraction of cytoplasmic actomyosin in its natural environment, i.e., still in association with regulatory control proteins. Detergents and glycerol, the common media used to permeabilize the plasmalemma, are accompanied by the disadvantages of an extensive deformation of cytoplasmic actomyosin fibrils as well as the difficulty to obtain defined degrees of extraction.

Advantages of cryosections as a new cell-free model include 1) the isotonic contraction of non-extracted fibrils can be directly observed after labelling with fluorescent phallotoxins and 2) the cryosection represents an open system allowing a reliable equilibration of the substrates, e.g., with respect to Ca^{++}, ATP and other compounds.

Due to the rather controversial role of Ca^{++} in cytoplasmic actomyosin contraction, this problem was investigated on both types of cell-free models derived from different stages of Physarum polycephalum:
MODEL I: Endoplasmic drops (300-2000 µm diameter) subjected to glycerol extraction for 20 h to several days demonstrate a reactivation reaction upon addition of Mg ATP

characterized by a volume change of the model. This reaction was registered by means of a microscopic photo-cell device.
MODEL II: Plasmodial strands experimentally enriched with "stress fibrils" were cryosectioned (2-4 µm) and stained with fluorescently labelled phallotoxins. The reactivation reaction is represented by an isotonic contraction of the fibrils and can be observed in the fluorescence microscope.

With respect to the identification of the role of Ca-ions in the contraction mechanism, both models and methods revealed identical results: Calcium prevents contraction in short-term, i.e., up to 20 h extracted specimens and non-extracted cryosections, whereas the inhibitory effect of calcium is lost after several days of extraction.

The inhibitory effect of calcium on the contraction mechanism of cytoplasmic actomyosin is supported by recent biochemical data (inhibition by binding of calcium to myosin LC-2). This fact may be of importance in the therapy of high blood pressure using Ca-antagonistic drugs.

For the discussion of the controversial findings concerned with the role of calcium in the control of cytoplasmic actomyosin contraction as well as for a comparison with muscle actomyosin, one has to consider the involvement of a complex actin equilibrium in the contraction of non-muscle cells and its control by calcium-sensitive, actin-modulating proteins, e.g., fragmin.

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428 ON THE ROLE OF THE CYTOSKELETON IN THE COMPARTMENTATION OF CARBOHYDRATE METABOLISM

Colin Masters, School of Science, Griffith University, Brisbane, Australia, 4111.

The common classification of glycolytic enzymes as 'soluble' reflects on their ease of extraction from cells and tissues rather than any realities of the cellular microenvironment. Indeed, there is an increasing emphasis in the literature on the complex and pervasive characteristics of the cytomatrical structures, and several studies have demonstrated that many of the enzymes involved in glycolysis are associated with the actin-based cytoskeletal systems in muscle and non-muscle cells, and that when these enzymes bind to cellular structure, substantial modifications in the kinetic characteristics may result (1,2). Furthermore, the extent of the interactions of these enzymes with cellular structure is dependent on the metabolic status of the cell, varies significantly during development and tissue differentiation, and may involve the formation of enzyme clusters by 'piggy-back' or secondary binding (2,3). Consequently the partitioning of these enzymes appears to offer a significant means of regulating metabolic activity, and one that provides for an appropriate positioning of an energy producing sequence in relation to the specific, dynamic requirements of the cytoskeleton (2). Additionally, however, there is increasing evidence of a reciprocal action of the glycolytic enzymes on the structure and function of actin (1,2). Aldolase and other glycolytic enzymes act to cross link actin filaments and alter the form of cytoskeletal elements, for example. It appears that the inter actions between glycolytic enzymes and the cytomatrix may provide not only a means of compartmenting carbohydrate metabolism, but also a means of modifying matrical structure.

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429 SUBCELLULAR LOCATION OF A 70KD POLYPEPTIDE MAY IMPLICATE ACTIN AS SUBSTRATUM FOR COATED VESICLE TRANSLOCATION.

D. Jane Bower and Peter Jeppesen MRC Clinical and Population Cytogenetics Unit, Western General Hospital, Crewe Road, Edinburgh, EH4 2XU, Scotland. A high proportion of scleroderma patients exhibiting the CREST syndrome (calcinosis, Raynaud's phenomenon, oesophageal dismotility, sclerodactyly, telangietasia)' possess a high circulating level of autoantibodies to a centromeric antigen which has been located on the trilaminar kinetochore plates. From a sample of three CREST patients possessing anti-kinetochore activity which we have examined, one, RW, also contained antibodies reacting with a cytoplasmic autoantigen. This antigen was shown by immunoblotting of SDS and 2-D gels to be a slightly basic polypeptide of 70KD. Indirect immunofluorescence to fibroblast monolayers showed that its distribution followed the actin stress fibres of the cells, in a punctuate pattern resembling that of coated vesicles. Indirect immunogold labelling of purified brain coated vesicles showed significant binding of serum RW to a minimum of 5% of the coated vesicle population. Transverse sections of fibroblasts incubated with the serum were significantly labelled with immunogold on the cytoskeletal network. Indirect immunofluorescence to fibroblast monolayers treated with either cytochalasin B for 1hr (which disrupts actin filaments) or colcemid for 20hrs, followed by 15mins extraction with 0.5% NP-40 (which removes detectable tubulin), showed that while tubulin removal had no visible effect on quantity or distribution of the autoantigen, disruption of actin filaments produced comparable changes in the distributions of the autoantigen and of actin filaments. The results show that the 70KD cytoplasmic autoantigen is associated with coated vesicles and with the actin filaments in the cytoskeleton, but not with tubulin. Since at least 5% of coated vesicles carry the antigen, this suggests that they too may be associated with actin filaments rather than tubulin in the cytoskeleton, and that the antigen may be associated with the actin filaments indirectly through its association with coated vesicles.

430 CARBOXY-TERMINAL REGIONS ON THE SURFACE OF TUBULIN AND MICROTUBULES

Frank Breitling and Melvyn Little. Institute of Cell and Tumor Biology, German Research Center, 6900 Heidelberg, FRG.

Tubulin peptides have been isolated reacting with the monoclonal antibodies YOL1/34, DM1A and DM1B. The relatively high reactivity of YOL1/34 with small peptides suggests that many of its determinants are within the overlapping regions of these peptides in positions α414-422. The smallest common region of peptides reacting with DM1A is α426-450 whereby the positions 426-430 appear to be particularly important for reactivity. Since it is known that the last few C-terminal residues are also accessible to enzymes and antibodies it seems that an extensive part (35-40 residues) of the acidic C-terminal domain is exposed on the surface of native tubulin dimers. In microtubules, however, the amino-terminal end of this region appears to be less accessible as YOL 1/34 reacts, poorly, if at all, with intact microtubules. (Kilmartin et al., 1982).

Peptides reacting with β-tubulin monoclonal antibody DM1B overlapped in positions β416-430. This indicates that β-tubulin is also positioned with at least part of its acidic C-terminal domain on the surface of microtubules, since DM1B reacts with unfixed microtubules after microinjection (Blose et al., 1984).

Lit.:

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431 TRANSFORMATION OF CURVED PROTOFILAMENT RIBBONS INTO MICROTUBULES DURING STEADY STATE.

K.J. Böhm, W. Vater, E. Unger. Academy of Sciences of the GDR, Central Institute of Microbiology and Experimental Therapy, DDR-6900 Jena, POB 73, GDR

In the presence of glycerol (6M) besides microtubules (MTs) protofilament (pf) ribbons with C- and S-shaped profiles are formed from brain microtubule protein. It has been shown that during steady state the percentage of aberrant assemblies, especially of C-shaped ribbons, is drastically reduced in favour of MTs.

Transformations of C-shaped ribbons into MTs might be realized in the following ways: a) Closing of ribbons with sufficient pf numbers. However, ribbons with pf numbers high enough to form MTs are seldom observed. b) Completing of ribbons by lateral association of further tubulin dimers or pfs, resulting in elevated mean pf numbers of the ribbon population. But in practice, this value remains unchanged, even after addition of "exogenous" tubulin dimers (equal amounts of polymerized and unpolymerized tubulin). c) Lateral association of two (or more) ribbons. Since most of the ribbons consist of 7-9 pfs the newly formed MTs should have 14-18 pfs. In contrast, the great majority of MTs has less than 14 pfs. d) Ribbons disassemble at their ends; freed tubulin dimers lengthen existing MTs. This mechanism agrees with the temporal constancy of the mean pf number of the ribbons. Moreover, it accords with observations that aberrant assemblies with 6-shaped profiles are also transformed into MTs and not into products with 8-shaped (and more complex) profiles.

The results provide further evidence for structural dynamics within populations of tubulin assemblies and support the thesis that at steady state C-shaped pf ribbons only after preceding disassembly may be converted into MTs. In the absence of MT-associated proteins no transformation events could be detected.

432 CHARACTERIZATION OF HIGH MOLECULAR WEIGHT MICROTUBULE-ASSOCIATED PROTEINS IN THE RAT PANCREAS. Jean-François Launay, Marie-Thérèse Vanier. INSERM, Unité de Biologie Cellulaire et de Physiopathologie Digestives, 67200, Strasbourg, France.

In the exocrine pancreas, it has been suggested that microtubules were implicated in the intracellular transport of exportable proteins (1). The subunit protein of microtubules, tubulin, has been characterized (2), purified from rat pancreas (3) and assembled into microtubules in presence of taxol (4). Several authors suggested that vesicular organelles are associated with microtubules in vivo, this association may be induced by some kind of microtubule-associated proteins (MAP's). The aim of this work was to demonstrate in the rat pancreas the existence of MAP's identified in brain as candidates for mediating the interaction of microtubules with cellular organelles. To isolated MAP's from rat pancreas, we have used the taxol-dependent method for purifying microtubules and MAP's. Microtubules were assembled from purified rat brain tubulin with the aid of taxol and were then added to a cytosolic extract of rat pancreas which did not support microtubule assembly from endogenous tubulin. Rat pancreas MAP's bound to the preformed microtubules and were isolated by centrifugation. MAP's were characterized by their electrophoretic mobility in SDS-PAGE and by immunoblotting experiments. These results demonstrated the existence in the rat pancreas of high molecular weight MAP's characteristic of brain tissue, suggesting that microtubules in neurons and secretory cells may be involved in at least some related functions. Moreover the availability of microtubules and MAP's will provide a useful tool for further in vitro investigation of interaction of microtubules with secretory granules.

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433 CHARACTERIZATION OF MAP-2 BINDING TO SYNAPTIC RAT BRAIN MITOCHONDRIA
Alvaro RENDON, Latifa DORBANI and Jean-François LETERRIER, Centre de Neurochimie du CNRS, Strasbourg, France

The purpose of our work is to study at the molecular level the interactions between microtubules and mitochondria. Isolated and well characterized rat brain synaptic mitochondria were prepared by a rapid Percoll gradient procedure. Thermostable ^{32}P-MAP-2 was obtained by autophosphorylation of rat brain microtubules followed by thermic denaturation of tubulin. ^{32}P-MAP-2 bind to synaptic mitochondria to a limited number of sites with an apparent Kd of (7.3 $\pm$ 1.0) 10^{-8}M. Comparatively synaptic plasma membranes and rat liver mitochondria show lower dissociation constants (one or two orders of magnitude respectively). The binding of MAP-2 to erythrocyte membranes was not saturable even at very large concentrations of ligand. The primary observations of this study are : a) Tubulin, BSA or holocytochrome-c do not compete for the binding of MAP-2 ; b) Purified high molecular weight subunits of neurofilaments partially inhibit the binding (50 % maximum inhibition) ; c) Non phosphorylated MAP-2 inhibits the binding of ^{32}P-MAP-2 more effectively that the non-radioactive phosphorylated protein ; d) A mitochondria associated protease degrades MAP-2 into several discrete polypeptides which do not bind to the organelle ; e) Digitonin subfractionation of mitochondria suggest an outer membrane localization of bound ^{32}P-MAP-2. Our results argue for specific interactions between brain synaptic mitochondria and microtubules mediated by MAP-2 association to the outer membrane and possible regulated by proteolysis. They also suggest the existence of binding sites for neurofilaments. Utilization of **in vitro** experimental systems such as those described here may help to elucidate the physiological role of the cross-link between microtubules, neurofilaments and mitochondria observed **in situ.**

434 THE EFFECT OF POLYAMINES ON TUBULIN ASSEMBLY. Peter Anderson (1) and Susan Bardócz (2). (1)Department of Biochemistry, Health Sciences, University of Ottawa, 451 Smyth Road, Ottawa, Ontario Canada K1H 8M5, and Department of Biochemistry, University Medical School of Debrecen, POB 6, H-4012, Debrecen, Hungary

Microtubules and polyamines are ubiquitous components of eucaryotic cells. It was found that assembly of microtubules is promoted by polyamines at physiological concentration. Cold, solubilized microtubules were prepared from calf brain by three cycles of polymerization-depolymerization. The rate of microtubule formation was detected by measuring the increase in turbidity at 350 nm. All polyamines tested increased the rate and extent of in vitro assembly of solubilized microtubules. The effectiveness of different polyamines in promoting polymerization was directly related to the number of free amino groups present in the polyamine. Spermine was the most effective with its four positively charged amino groups. Spermidine and N^1-acetylspermine was less effective than spermine, but more effective than putrescine or N^8-acetylspermidine. The presence of Ca^{2+} or cholchinine inhibits the microtubule assembly. Microtubule formation in vivo may be controlled by polyamines present in the cell.

435 HETEROGENEITY OF MICROTUBULES STUDIED BY MONOCLONAL ANTIBODIES TO ALPHA-TUBULIN

Pavel Dráber (1), Eduarda Dráberová (1), Vladimír Viklický (1), Piero Cappuccinelli (2). (1) Institute of Molecular Genetics, Czechoslovak Academy of Sciences, Vídeňská 1083, 142 20 Prague 4, Czechoslovakia and (2) Institute of Medical Microbiology, University of Sassari, Viale S. Pietro, 07100 Sassari, Italy.

A panel of four monoclonal antibodies (TU-01, TU-02, TU-03, TU-04) was prepared against pig brain tubulin. All four antibodies recognized an antigenic determinant on the alpha-subunit of brain tubulin from different species. From the results of immunoblotiing of digested alpha-tubulin it was possible to identify three types of reactivity within these antibodies. The immunoreactivity of tubulins of different origin was studied by immunofluorescence and immunoblotting. The recognized antigenic determinants were widely distributed among different cells and animal species of higher eukaryotes but were absent in some lower eukaryotic cells. In addition, the monoclonal antibodies discriminated between different microtubules or parts of them within individual cells. The highest degree of heterogeneity was found in protozoan cells. The prepared monoclonal antibodies can serve as a useful tool for the screening of the differences in microtubules and for studying the functional and chemical heterogeneity of tubulins.

436 ULTRASTRUCTURAL GOLD DOUBLE LABELLING OF MOUSE SPERMATOCYTE MICROTUBULES:

I.Raška(1),P.Valouch(2),V.Petrašovičová(2),P.Dráber(3),V.Viklický(3),M.Jarník(1), J.Lejnar(1),Z.Kostrouch(4).(1)Inst.Exptl.Med,Czech Acad Sci,Lidových milicí 61,Prague (2)Res.Inst.Rheumatology,Na slupi 4,Prague,(3)Inst.Molecular Genet.,Czech Acad Sci, Vídenská 1083,Prague,(4)Inst.Pathol-anat.,Charles Univ.,Studničkova 2,Prague,ČSSR.

A preliminary immunofluorescent study of mouse spermatocyte microtubules was performed with the help of two different anti-tubulin monoclonal antibodies,the antibody TuO1 (IgG $\varkappa$) and antibody TuO2 (IgM $\varkappa$).These two antibodies exhibit affinities to two different epitopes on alpha-tubulin.The light microscopical study pointed to a heterogenous staining pattern of mouse spermatocyte by these monoclonal antibodies.In order to get a deeper and more clear cut insight into such a heterogenous immunostaining picture of microtubules,we have performed in this study the ultrastructural localization of alpha-tubulin by means of antibody colloidal gold complexes.The post embedding labelling procedure was carried out on thin sections of aldehyde fixed and Lowicryl K4M embedded cells.Gold complexes were formed with gold particles of two different sizes,roughly 9 nm and 18 nm large.The following macromolecules,used to stabilize both kinds of gold particles,were included in the labelling protocol: TuO1, TuO2, unrelated monoclonal antibodies of IgG or IgM subclasses and bovine serum albumin. Control experiments included also preadsorption of thin sections to free immunoglobulins.For the double labelling procedure,consecutive as well as concomitant exposure of sections to complexes gold-TuO1 and gold-TuO2,were performed.When analysing the labelling data,special care was paid to the influence of the following parameters: accessibility of the epitopes,influence of aldehyde fixation and Lowicryl embedding, influence of the cutting process.

437 BEHAVIOUR OF MICROTUBULES IS COUPLED WITH CHROMOSOME CONDENSATION ACTIVITY IN HYBRIDS BETWEEN MOUSE MEIOTIC OOCYTES AND INTERPHASE BLASTOMERES.

Jacek Kubiak,Dept.of Embryology,Inst.of Zoology,Warsaw University,Warszawa,Poland

Mouse interphase blastomeres from 2,4,8-cell embryos were electrofused with ovulated oocytes arrested in metaphase II.The hybrids were double-stained for microtubules /MTs//indirect immunofluorescence/ and DNA /propidium iodide/.

Interphase network of MTs undergoes rapid disassembly 20 min.after fusion.Similar behaviour of interphase MTs was observed when somatic cells /MO giant cells/ were substituted for blastomeres. This result indicates that metaphase cytoplasm posseses properties favouring spindle formation and disassembly of interphase MTs. Later on after fusion introduced nuclei started prophase-like changes /prophasing/ i.e. the begining of premature chromosome condensation /PCC/. Prophasing nuclei were surrounded by astral MTs forming frames around them. Concomitantly astral MTs appear in the hybrid cytoplasm. After nuclear envelope breakdown spindle-like structures or multipolar spindles form around PCC. This indicates that PCC,despite unfunctional kinetochores /Szollosi et al.1980;in press/can organize spindle-like structures. In the cytoplasm of such hybrids particulary high number of cytoplasmic asters appear. Additionally new MTs appear within the spindle transforming it into astral one. When oocyte-derived parts of hybrids undergo activation by the time of fusion no changes of introduced nuclei and no MTs around them were observed.

We suggest that the observed behaviour of MTs can be attributed to high critical concentration for tubulin assembly in metaphase cytoplasm and low in interphase one.

438 ***MICROTUBULES DISTRIBUTION DURING MOUSE OOCYTE MEIOTIC MATURATION. EFFECT OF TAXOL.***

H. RIME, C. JESSUS, R. OZON. *Laboratoire de Physiologie de la Reproduction (UA CNRS 555) Université Pierre et Marie Curie, 4 Place Jussieu, 75252 Paris Cédex 05, France.*

The Mammalian oocyte is normally arrested in the dictyate stage of the first meiotic division. It resumes meiosis spontaneously in vitro when isolated from its follicular environment.

To explore the spatial distribution of microtubules during meiotic maturation, we have performed anti-Tubulin and anti-MAP1 (gift of M. DE BRABANDER) immunofluorescence which recognizes MTOC in somatic cells.

At the germinal vesicle stage, anti-Tubulin antibody reveals a high background staining in the oocyte cytoplasm. In contrast anti-MAP1 antibody stains exclusively the nucleoplasm. When the nuclear envelope breaks down (2-3 h of culture) numerous microtubules arrays appear in the nucleoplasm/cytoplasm region.

These microtubules arrays are decorated by anti-MAP1 antibody. After 10-12 h of culture the antigene component detected with anti-MAP1 antibody is present at the poles of the meiotic spindle.

The antimitotic agent : Taxol (10 μM) induces microtubules formation at the periphery of the germinal vesicule. At GVBD period numerous cytoplasmic asters now appear in the cytoplasm. These data suggest that the critical concentration for tubulin polymerisation decreases during the meiotic maturation process in mouse oocyte.

439 ***MICROTUBULES AND MICROTUBULE-ASSOCIATED PROTEINS DURING MEIOTIC MATURATION OF XENOPUS OOCYTE***

C. JESSUS, D. HUCHON and R. OZON. Laboratoire de Physiologie de la Reproduction - Université Pierre et Marie Curie - 4, Place Jussieu 75252 Paris Cédex 05, France.

Microtubules and microtubule-associated proteins (MAPs) have been visualized by immunocytological method at different steps of the meiotic maturation process of the Xenopus oocyte. In the prophase-blocked oocyte, only few microtubules are present at the level of the upper part of the nucleus. At this stage of oocyte development, a monoclonal anti-MAP1 antibody (an antibody which specifically stains microtubule-organizing Center (MTOC) in mammalian somatic cells ; a gift of M. DE BRABANDER, Belgium) reveals a punctuate staining in nuclei and the nucleoli, but not the perinuclear microtubules. During progesterone induced maturation, the perinuclear microtubules of the prophase stage depolymerize and a new microtubular array appears at the basal part of the disrupting nuclear envelope ; anti-MAP1 antibody now stains a dense layer located immediatly below this array. This transitory network preceeds the formation of the two metaphase spindles. These results indicate that microtubules are specially reorganized during the transition prophase-metaphase of the Xenopus oocyte. In order to further analyse the mechanisms controlling microtubule assembly at these two stages of the first meiotic division, taxol was microinjected :

1) in prophase-blocked oocyte, taxol is unable to induce microtubule elongation or polymerization (no aster) ;

2) in matured oocyte as soon as the nuclear envelope breaks down, taxol now promotes microtubule assembly since numerous asters are now observed. When the oocytes are treated with anti-MAP1 antibody the center of the asters are hightly stained.

We proposed that the nuclear antigen which is recognized by anti-MAP1 antibody, is released into the cytoplasm at the onset of the nuclear breakdown and that it represents a regulatory factor necessary for microtubule assembly in the cytoplasm.

440 CHANGES IN MICROTUBULAR SYSTEM OF MURINE IMMUNOCYTES UPON ANTIGENIC ACTIVATION.

Petr Šíma, Martin Bilej. Institute of Microbiology, Czechoslovak Academy of Sciences, 142 20 Prague 4, Czechoslovakia

We determined the characteristic morphological pattern typical for the organization of microtubular systems of murine macrophages and lymphoid spleen cells. During in vitro cultivation in non-antigenic conditions up to 48 h a stable part of both macrophages (about 10 %) and lymphoid cells (about 25 %) formed prominent microtubular extremities (uropods). On the other hand, if the murine cells were cultivated for the same time in the presence of Salmonella typhimurium (a particular antigen) or lipopolysaccharide (a soluble antigen), the number of cells with prominent extremities (uropods) increased at 48 h up to 90 % in macrophages and 40 % in lymphoid cells. After in vivo presensitization of animals with Salmonella typhimurium, the reorganization of microtubuli took place earlier in both the non-antigenic and the antigenic environment. The macrophages and lymphoid cells with prominent microtubular extremities (uropods) formed on average 30 % of population already after 2 h of cultivation. At 48 h the percentage of cells with reorganized microtubular system reached the same values (90 % of macrophages and 40 % of lymphoid cells) as in the case of cells taken from non-presensitized mice and cultivated in antigenic conditions.

441 QUANTITATIVE ANALYSIS OF TUBULIN AND MICROTUBULES IN CHOLERA TOXIN AND PERTUSSIS TOXIN-TREATED CHINESE HAMSTER OVARY CELLS

Scapigliati G., Rappuoli R., Bartalini M. and Pallini V.
SCLAVO RESEARCH CENTRE Via Fiorentina 53100 SIENA - ITALY

Cultures of mammalian cells treated with cholera toxin (CT) undergo several physiological and morphological changes including accumulation of intracellular cAMP and stabilization of the microtubules (MTs) (1). The distribution of soluble tubulin, MTs and microtubule associated proteins (MAPs) in the cytoplasmic and cytoskeletal fractions of CT and pertussis toxin (PT)-treated CHO cells was studied by using indirect immunofluorescence (IIF), colchicine-binding filter assay and high resolution 2D gel electrophoresis, the cells were extracted in conditions that preserve preformed MTs. Here we report that the stabilization of MTs induced by 10 ng/ml of CT is clearly observed in IIF of extracted cells and in the 2D gels pattern of cytoplasmic and microtubular fractions. The maximal stabilization of MTs with respect to tubulin (2,5 times) is reached after two hours of treatment and this ratio is maintained if CT is not removed; this kinetic is similar to that of accumulation of intracellular cAMP. Cells treated with PT at the same concentration do not show any alteration of the ratio MTs/tubulin, however the pattern of MTs observed by IIF is different.

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442 ROLE OF CYTOPLASMIC MICROTUBULES IN YEAST CELL CYCLE

Jiří Hašek, Jana Svobodová, Eva Streiblová. Institute of Microbiology, Czechoslovak Academy of Sciences, 142 20 Prague 4, Czechoslovakia

The display of cytoplasmic microtubules was followed in cells and protoplasts of Saccharomyces uvarum using indirect immunofluorescence with monoclonal antitubulin antibody TuO1. The dynamics of microtubule rearrangements was observed in relation to cell wall removal. The differential effect of mitotic inhibitors on the yeast microtubule complement was demonstrated. The role of cytoplasmic microtubules in control of the yeast cell cycle is discussed.

443 EFFECTS OF ALLOXAN, Zn^{2+} AND ZINC CHELATES OF ALLOXAN ON MICROTUBULES ASSEMBLY AND INSULIN SECRETION

Rainer Schmidt (1), H. Müller (2), P. Glaß (1), S. Schneider (1), and E. Unger (2), (1) Institute of Anatomy, Martin-Luther-University, GDR-4020 Halle, POB 302, and (2) Department for Experimental Cell Research, Institute of Microbiology and Experimental Therapy, Academy of Sciences of the GDR, GDR-6900 Jena, Beutenbergstraße 11

The mechanism of the diabetogenic action of alloxan is still unknown, although observations suggest that the intracellular formation of a zinc chelate of alloxan may be the important step in producing blood sugar increases by preventing the decomposition of alloxan into non-diabetogenic alloxan acid. Alloxan, however, is one of the poisons causing disturbances in the mitosis of the islet cells also. These characteristics might affect assembly of microtubules (MT) and modify secretion in suggesting a role of MT in the slow release of insulin secretion. We studied the effects of alloxan and Zn^{2+} on MT and MT assembly *in vitro* as well as on blood sugar values and disturbances in the mitosis of B-cells following doses of alloxan and zinc alloxan chelate i.v. (70 and 35 mg/kg b.w.). The results reveal that alloxan inhibits MT assembly *in vitro* and brain tubulin assembles into flat sheets, one type of abnormal assembly, when Zn^{2+} are present. A higher percentage of disturbances of B-cell mitoses *in vivo* may be caused by an attack of alloxan on MT but the blood sugar level (alloxan induced hypoglycaemia 6 h p.i., permanent hyperglycaemia 28 h later, zinc alloxan chelate hyperglycaemia without preceeding hypoglycaemia) and the instantaneous destruction of B-cell secretory granula, where the chelate formation *in vivo* takes place generally, may have other, pharmacological, chemical, or cytotoxic effects, unrelated to MT.

444 HIGH RESOLUTION AUTORADIOGRAPHIC STUDIES ON THE INCORPORATION OF ^{3}H PALMITIC ACID INTO ORNITHOGALUM UMBELLATUM'S LIPOTUBULOIDS CONSISTING OF LIPID DROPLETS SURROUNDED BY A SYSTEM OF MICROTUBULES.

Maria Kwiatkowska. Department of Plant Cytology and Cytochemistry, Institute of Physiology and Cytology, University of Łódź, 90-237 Łódź, Poland

In the ovary epidermis of O. umbellatum lipotubuloids were found, that is, an agglomeration of lipid droplets surrounded by a half unit membrane, entwined and held together by a system of microtubules /PROTOPLASMA 75: 345-357; 77: 473-476/. Using light and EM-autoradiography with the 2 h incubation of ^{3}H palmitic acid 25 uCi/ml it was found that lipotubuloids are a site of intense incorporation of this isotope. After extraction of lipids with lipid solvent the lipotubuloids were not labelled. Localization of autoradiographic grains after 15 h postincubation with isotope-free medium indicates a migration of the labelled material from the lipotubuloids to the whole cells. Ultrathin studies demonstrated that after 2 h incubation 52% of autoradiographic grains were localized over the site of the microtubules adjoining closely the half unit membranes of lipid droplets, whereas 6% of grains were found on the centre of lipid droplets; 27% of grains were placed over the microtubule bundles combining the lipid droplets, 15% of grains - over the cytoplasm of lipotubuloids. The possible connections of the microtubules with lipid synthesis in the lipotubuloids are discussed.

445 MICROTUBULE ULTRASTRUCTURE IN DERMAL MELANOPHORES AND SPINAL NERVE OF THE ANTARCTIC TELEOST PAGOTHENIA BORCHGREVINKI (NOTOTHENIIDAE, PERCIFORMES).

Masataka Obika (1), V. Benno Meyer-Rochow (2). (1) Department of Biology, Keio University, Yokohama, 223, Japan, and (2) Department of Biological Sciences, University of Waikato, Hamilton, New Zealand.

The antarctic teleost Pagothenia borchgrevinki inhabits the Antarctic Ocean where the water temperature remains around -1.9°C throughout the year. Dermal melanophores of this fish respond within minutes to adrenaline and theophylline with melanosome aggregation and dispersion, respectively. Numerous cytoplasmic microtubules are present in these cells in spite of low environmental, temperatures. In longitudinal profiles, many microtubules are twisted, beaded and sometimes even branched. In cross sections 'C', 'U', 'S', '6', and other irregularly-shaped tubules are observed. Nocodazole partially disrupts microtubules and inhibits adrenaline-induced pigment aggregation. Pigment movement is also prevented by erythro-9-[-3-(2-hydroxynonyl)]-7-adenine. Although the participation of these incomplete microtubules in cell motility remains uncertain, the results indicate that this fish has a cold-resistant microtubular system on which melanosome movements depend. Unlike those in the melanophores microtubules in the axons of spinal nerves are of uniform thickness and often contain an electron-dense core in the centre.

446 SUPERNUMERARY MICROTUBULES IN AXONEMES OF SENSORY CILIA IN A CILIATE DILEPTUS ANSER.

Krystyna Golińska, Nencki Institute of Experimental Biology, Polish Academy of Science, 02-093 Warsaw, Poland.

The so-called sensory cilia in Dileptus are short and arranged in pairs on the anterior-dorsal side of the cell. Microtubule pattern within the sensory cilia is 9 + 2, which is different from the pattern in locomotor cilium in lack of B subfibre on 9 microtubules, and in the absence of intertubular links. The supernumerary microtubules were observed in 10% out of 120 cross sections of sensory shafts, reulting in 10 + 2 and 11 + 2 microtubule patterns. Further increase in microtubule number, up to 14 + 2 pattern, was found in 30% out of 80 cross sections of sensory axonemes grown in elevated temperature. The supernumerary microtubules were found only within the ciliary shafts, while the basal bodies and transition regions of these cilia showed no structural aberration. The additional microtubules seem to grow out of clusters of dense material, which may contact the ciliary membrane.

It is suggested that the supernumerary microtubules are the remnants of locomotor ciliary shaft, since the posterior sensory cilium of each pair grows on the basal body that has previously been equipped with locomotor cilium. Temperature-induced increase in microtubule number within sensory shafts may reflect the increased imperfection in separation of the locomotor shaft from its basal body during formation of sensory unit, and/or a defective resorption of the locomotor shaft.

447 ISOLATION OF CENTROSOMES FROM A HUMAN CELL LINE. M. Bornens (1), J. Berges (1), M. Paintrand, E. Karsenti (2). C.B.C. CNRS, 94200 Ivry-sur-Seine (France). (1) Present address : C.G.M. CNRS, 91190 Gif-sur-Yvette (France). (2) E.M.B.L., Heidelberg (Germany).

An isolation procedure, modified from Mitchison et al (1), was developed on the human lymphoblastic KE 37 cells, which provides high yields of centrosomes, capable of promoting tubulin assembly. The main modifications were the addition of a cocktail of antiproteases during the lysis, a Dnase II treatment of the lysate supernatant and a direct sedimentation of the lysate on a discontinuous sucrose gradient. Centrosomes were recovered in their native paired configuration. They were virtualy free of any contaminant as juged by phase contrast microscopy, by the absence of staining with the dye Hoechst 33258, known for its high sensitivity towards nucleic acids that are the major potential contaminants of this preparation and by electron microscopy. Centrioles in a pair appear linked by fibers. A proximo-distal organization of each centriole can be observed with the following features : the lumen at the proximal side appears empty whereas a complex structure around an axial hub of about 40 nm in diameter can be observed filling the lumen at the distal end. The periphery of each centriole is covered at the proximal end with a thick layer (about 0.1 µm) of osmiophilic material whereas the distal side demonstrates 9 long appendages distributed with a radial symmetry. The average amount of protein per centrosome is 3.10^{-4} pg. The polypeptide pattern appears quite specific when compared to either detergent-soluble or -insoluble cellular proteins. It is characterized by two major groups of proteins, one of high molecular weight and the other in the range of 50 to 65 kDa. Western-blotting with an anticentrosome serum allowed us to demonstrate a dramatic enrichment of centrosomal components in such preparations.

(1). Mitchison T. and Kirschner M., 1984. Nature, 312, 232-237.

448 THE CENTROSOME AND ITS ROLE IN THE ORGANIZATION OF MICROTUBULES.

Ivan A. Vorobjev. A.N.Belozersky Laboratory of Molecular Biology & Bioorganic Chemistry, Moscow State University, 119899 Moscow, USSR

A study was made: 1) On the dynamics of microtubules reconstituted in the PE cells after cooling and colcemid treatment. The number of microtubules radiating from the centrosome reaches a maximum 15 min after the cells are placed again at 37°C, and 35 min after removing colcemid from the culture medium. In both cases the number of microtubules attached to the centrosome decreases subsequently. 2) On the number of microtubules radiating from the centrosome in spreading fibroblasts. This number also appears to be inconstant. The maximal number is at the time of radial spreading, and it slightly decreases in polarized cells. In a monolayer culture the number of microtubules decreases significantly.

It is suggested that two systems of microtubules exist in a cell. One forms a network in the cytoplasm and has no definite convergence foci. The other radiates from the centrosome. It is presumed that the radiating microtubules persist bound to the centrosome only for a definite time interval during their growth. Subsequently, after their growth is completed the microtubules disengage and depart from the centrosome. New microtubules may start growing on the centrosome. Consequently, the centrosome operates as a microtubules producing conveyer.

449 IDENTIFICATION OF CENTROSOMAL PROTEINS IN A HUMAN LYMPHOBLASTIC CELL LINE : ANTIGENIC RELATIONSHIP WITH HUMAN LACTATE DESHYDROGENASE. F. Gosti, M.C. Marty, J.C. Courvalin, M. Bornens. Centre de Génétique Moléculaire, 2 avenue de la Terrasse, 91190 Gif-Sur-Yvette, France, tel 69.07.78.28.

A non-immune rabbit serum (0013) reported for its strong human specificity was used for the identification of pericentriolar proteins in the human T-lymphoblastic cell line. In a highly enriched preparation of centrosomes, the major antigens consisted of a family of high molecular weight proteins (130 kDa and 180 kDa to 250 kDa) together with a doublet of 65/60 kDa. As demonstrated by ultrastructural localization in situ and protein analysis of isolated centrosomes, these antigens are abundant components of the pericentriolar material where MTOC activity is localized. They also redistribute as expected for a nucleating or capping protein in physiological or experimental conditions where centrosome is no longer acting as a nucleation center. This together with the cyclic change of centrosomal staining with cell cycle, suggests the involvment of 0013-specific antigens in microtubule nucleation. The serum 0013 was however complex as evidenced by its specificity towards other cytological structures and by its interaction with several non centrosomal proteins. Using several independent cell fractionation procedures and affinity purification of Igs on antigens the serum was demonstrated to exhibit a minor specificity, concerning a protein of 75 kDa likely to be membrane-associated, and a major specificity corresponding to the centrosomal antigens, to a 80 kDa nucleolar protein, and surprisingly, to an abundant cytosolic protein identified as the human lactate deshydrogenase. The evolutionary and physiological significance of this situation is under investigations.

450 CENTRIOLAR STRUCTURE AND THE ROLE OF CENTROSOMES IN MULTINUCLEATE CELLS.

Dieter Schroeter (1), Neidhard Paweletz (1), Sibdas Ghosh (2). (1) Institute of Cell and Tumor Biology, German Cancer Research Center, Im Neuenheimer Feld 280, D-6900 Heidelberg, Federal Republic of Germany, (2) Dept. Botany, University of Calcutta, India.

Multinucleate PtK-1 or HeLa cells develop spontaneously in small amounts (< 0.1 %) or can be induced by application of antimitotic drugs or fusion of mononucleate cells. We studied the genesis and further development during cytokinesis of such cells in the light as well as in the electron microscope. We observed that multinucleate cells can divide again forming: (i) multipolar spindles with all chromosomes, (ii) unsynchroneously developing spindles of different nuclei or groups of nuclei., and (iii) synchroneously but separately developing spindles for each nucleus. The observations were focussed on the behaviour of centrioles and membrane distribution patterns, since anormalities of microtubular arrangements in the half spindles and of the kinetochores could not be seen. Our preliminary results indicate that in multinucleate cells in interphase the number of centrioles is increased but more or less independent from the number of nuclei. Additional centrioles can form satellites, basal body structures, cilia, daughter centrioles, ciliary roots or similar fibrillar arrangements. Mostly they are clustered near the periphery of the cell in a relatively long distance from the nuclei. During spindle pole formation all additional centriolar products have disappeared. A minimum of two to three half spindles (spindle poles) contain a normal duplicate set of centrioles and exhibit complete centrosomes. These centrioles never were observed to have additional structures. The other half spindles need not to contain centrioles. It has to be discussed whether or not at least 2 complete spindle poles are necessary to raise a higher order of orientation during cell division and to orientate the plane of cleavage.

451 GAMMA-AMINOBUTYRIC ACID (GABA)-LIKE IMMUNOREACTIVITY IN KINETOSOMES OF THE RAT OVIDUCTAL CILIA.

S.L.Erdő (1), J.Somogyi (2), J.Hámori (2) (1) Pharmacological Research Centre, Chemical Works of Gedeon Richter Ltd., P.O.Box 27, H-1475 Budapest, Hungary, (2) 1st Department of Anatomy, Semmelweis University Medical School, H-1450 Budapest, Hungary

Gamma-aminobutyric acid (GABA) a recognized inhibitory neurotransmitter in the vertebrate central nervous system, has recently been demonstrated in extremely high concentrations in the rat oviduct. Considering the relatively poor innervation of the oviduct, it seemed unlikely that the high amount of GABA in the oviduct was located exclusively in neurons. The present study is an attempt to investigate the cellular and subcellular distribution of GABA in the rat oviduct with a specific anti-GABA serum prepared by Hodgson et al. (J.Histochem.Cytochem. 33, 229, 1985). Sections of the oviduct were incubated in the presence of the anti-GABA serum (1000 fold dilution) for 90 min, then processed for immunocytochemical evaluation as described by Somogyi et al. (J.Histochem.Cytochem. 33,240, 1985). At light-microscopic level, specific immunoreactivity was present primarily at the ciliated epithelial surface of the oviduct. Electron-microscopic evaluation of the epithelial area revealed that GABA-like immunoreactivity is confined to the basal bodies (kinetosomes) of the cilia, while cilia themselves show only a faint staining. At higher magnification, specific immunoreactivity was found to be confined to the tubular elements of the basal bodies. These results indicate that GABA in the rat oviduct is located extraneuronally. Thus, GABA may have a function other than neurotransmission. The occurrence of the GABA-like immunoreactivity in the basal bodies of the cilia suggests a possible role for GABA in the ciliary functions.

452 CALCIUM CONTROL OF CILIARY BEAT : THE EFFECTS OF LINDANE, A POTENT INSECTICIDE.

F. MARANO (1), S. KRISHNASWAMY (1), H. BERREBAH (1), C. BETTRENCOURT (2) and B. VOLOCHINE (2).

(1) Laboratoire de Cytophysiologie végétale et de Toxicologie cellulaire, UA CNRS 567, Université PARIS VII, 2 Place Jussieu, 75005 PARIS, France.

(2) Laboratoire de Diffusion inélastique de la Lumière, Université PARIS V, 45 rue des Saints Pères, 75005 PARIS, France.

The ciliary beat and cell motility of *Dunaliella* and *Chlamydomonas* (biflagellate green algae) has been studied using Laser Doppler velocimetry (LDV) and high speed microcinematography. We have shown that cell velocity could be well estimated by means of LDV which allows velocity measurements in less than 2 min.

Lindane (γ-hexachlorocyclohexane), a largely used insecticide, has a very rapid, dose-dependent cilio-inhibitory effect on the two kinds of biflagellate species. Three other isomers, α, β and δ -HCH were tested and it appears that there would be a relation between insecticide power and cilio-inhibitory effect.

High speed microcinematography showed that not all the phases of ciliary beat were uniformly affected by the drug : only the effective stroke was greatly slowed down by lindane. Lindane is known to interact with calcium homeostasis, so data obtained by LDV after treatment by lindane in a medium without calcium or with a ionophore A23187 suggest that the drug could induce the strong inhibition of ciliary beat by an increase of cytoplasmic calcium.

453 NUMBER OF CILIARY ROWS AND THEIR REGULATION IN SMALL FRAGMENTS OF A CILIATE DILEPTUS ANSER.

Jolanta Drzewińska, Nencki Institute of Experimental Biology, Polish Academy of Sciences, 02-093 Warsaw, Poland.

The number of ciliary rows was studied in cells belonging to three groups. The first group were control cells taken from a growing culture. The second and third group were small cells obtained after regeneration of slices cut off from the middle of the Dileptus body; thus the initial number of ciliary rows was the same in all groups of cells. Dileptuses from the second group were fixed 24 hrs after the operation, those from the third group were fixed 48 hrs after the operation. Since the regenerating cells were kept in a medium deprived of food, a very tiny cells resulted.

In comparison to control cells, dileptuses from the second group had the circumference of their bodies diminished, while the number of ciliary rows remained the same. Cells from the third group showed a further decrease in the circumference of cell body, alongside with its elongation, and the decreased number of ciliary rows. Only the dileptuses from the first and third groups had the circumference of their bodies and the number of ciliary rows positively correlated.

It is concluded that the downward regulation of the number of ciliary rows takes place sometime between 24 and 48 hours after the operation.

454 SUPERNUMERARY ELEMENTS OF CILIARY PATTERN IN mlm MUTANT OF PARAUROSTYLA WEISSEI /CILIATA/ AND THEIR DEVELOPMENT DURING MORPHOGENESIS.

Bożena Dubielecka Nencki Institute of Experimental Biology, Polish Academy of Sciences, 02-093 Warsaw, Poland

Ciliary pattern in wild-type morphostatic cells of P. weissei contains only one row of the left marginal cirri /LMC/, while an expression of mlm mutation brings about an increase in number of LMC up to 8 rows. The wild-type primordia of LMC arise as a result of disaggregation of the preexisting cirri in the old LMC during morphogenesis. Formation of LMC primordia in mlm mutant may occur without participation of the old structure, preferably in the areas close to forming oral apparatus. The effect of the mlm mutation involves also a proliferation of excess basal bodies. The observed overproduction of the basal bodies is resticed to the right portion of the LMC domain and to the left portion of the oral apparatus. It is concluded that the area controlled by mlm gene does not coincide with a well defined region of ciliary pattern in P. weissei.

455 SELF-ASSEMBLY INTERMEDIATES OF PLECTIN.

Roland Foisner, Georg Weitzer and Gerhard Wiche, Inst.Biochem., Univ.Vienna, Austria.

Plectin (300 kDa) was purified from detergent/high salt resistant intermediate filament preparations of rat glioma C_6 cells by solubilization in 7M urea and subsequent gel permeation chromatography. Transmission electron microscopy of uranyl acetate stained plectin samples desalted into low ionic strength buffer revealed globular or slightly elliptical structures (20-30nm diameter) and aggregates of 2-10 globes. In physiological buffers large bulky or elongated aggregates of these globes were the predominant structures. Plectin structures of similar appearance were observed when ionic detergents instead of urea were used for solubilization. To reveal the molecular structure of plectin more clearly, urea solubilized protein samples were transferred to various buffers and examined by rotary shadowing electron microscopy. At protein concentrations lower than 30µg/ml of low ionic strength buffers and at 4°C, short rods (2-5nm diameter, up to 50nm length) and globular particles (5-10nm) were the predominant structures observed; whereas, 5nm thick filaments up to 200nm in length, often displaying globular particles at one or both ends, were found at protein concentrations of 100µg/ml. These filaments formed higher star-like aggregates upon increasing the ionic strength of the buffer solutions, seemingly by binding at their globular heads. The star-like plectin structures displayed a globular central region of about 50nm diameter and numerous filamentous 2-5nm thick extensions 50-200nm long. Often aggregates of star-like structures interlinked by their filamentous extensions were found. The aggregation of plectin was followed also by turbidimetric measurements. The Stokes radii of plectin structures in low ionic strength buffers determind by HPLC confirmed the electron microscopic data.

456 INTERACTION IN VITRO OF INTERMEDIATE FILAMENTS AND THEIR SUBUNIT PROTEINS WITH LIPID VESICLES. Georgios Perides, Peter Traub, Max-Planck-Institute for Cell Biology, Rosenhof, D-6802 Ladenburg/Heidelberg, FRG

The interaction of intermediate filament proteins (IFPs) with lipids from Ehrlich ascites tumor cells, individual phospholipids, and a mixture thereof, has been investigated. At low as well as at physiological ionic strength, the complexes formed had densities in sucrose gradients distinctly different from those of the original reactants. In KBr density gradient equilibrium centrifugation, the IFP-lipid adducts accumulated as thin proteolipid films on top of the KBr gradients, whereas in the absence of lipids the proteins were distributed within the density gradients. The affinity of IFPs for negatively charged phospholipids was substantially greater than that for vesicles produced from uncharged phospholipids. Limited digestion of IFPs with various proteinases demonstrated that for optimal association of the reactants the IFPs must carry an intact N-terminus and that the N-terminal polypeptide (95 amino acids) itself shows strong reactivity with lipid vesicles. Analysis of the extent of formation and stability of IFs by sucrose gradient centrifugation and electron microscopy revealed strong inhibitory effects by vesicles prepared from negatively charged phospholipids, whereas liposomes derived from uncharged phospholipids were virtually ineffective. Intermediate effects were observed with vesicles produced from phospholipid mixtures with compositions characteristic of mammalian cells. These relationships are interpreted as a possible molecular basis of the association of non-epithelial IFs with natural membrane systems such as the plasma membrane, the nuclear membrane, the endoplasmic reticulum, mitochondria and other membrane-bounded structures of the cytoplasm.

457 COEXPRESSION OF DESMIN AND VIMENTIN IN LOW GRADE RHABDOMYOSARCOMA. IMMUNOHISTOCHEMICAL, BIOCHEMICAL AND ULTRASTRUCTURAL INVESTIGATION

Leoncini P. (1), Bugnoli M. (1), Scapigliati G. (1), Leoncini L. (2), Cintorino M. (2)
(1) SCLAVO Research Centre, Via Fiorentina 1 - 53100 Siena - ITALY
(2) Dept. of Pathology, University of Siena - ITALY

The utility of desmin as a marker of muscle tissue origin has been recently confirmed. We report a case of poorly differentiated mesenchymal tumor of the orbit, in which immunohistochemical investigation performed on formalin-fixed and paraffin-embedded sections showed the presence of desmin and vimentin in the same cells; ultrastructural analysis had previously suggested an undifferentiated mesenchymal tumor without any sign of myogenic differentiation. These results demonstrate the utility of intermediate filament typing in tumor diagnosis, which is highly convalidated by correlation of immunohistochemical with biochemical investigation; moreover, the expression of desmin and vimentin with distinctive features in a morphologically poorly differentiated mesenchymal neoplasia may suggest that the tumor originates from a primitive cell "committed" to myogenic differentiation.

458 CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST VIMENTIN

V. Viklický (1), P. Dráber (1) and F. Havlíček (2). (1) Institute of Molecular Genetics, Czechoslovak Academy of Sciences, Vídeňská 1083, 142 20 Prague 4, Czechoslovakia and (2) 1st Department of Pathological Anatomy, Charles University, Prague, Czechoslovakia

A panel of monoclonal antibodies (VI-01 - VI-05) to intermediate filaments of vimentin type has been isolated from fusions of mouse myeloma cells with immunoblot technique using whole cell extracts. In spite of the complexity of the transferred patterns, only the 57 kilodalton (KD) band at the position of vimentin was recognized by VI-01, VI-02 and VI-03 antibodies in LEP cells. Some additional bands were labelled with antibodies VI-04 and VI-05. In BHK-21 cells the binding of all antibodies occurred on the 57 KD band and on lower molecular weight bands. All five antibodies reacted with purified vimentin and desmin. In cryostat sections of normal adult rabbit tongue, small intestine, kidney, uterus, liver and testes, VI-01, VI-02, VI-03 and VI-05 antibodies stained fibroblasts. Endothelial and sooth muscle cells of blood vessels were stained by VI-01, VI-03 and VI-05 antibodies. Positive reactivity in epithelial cells was seen with the VI-03 antibody. Antibodies VI-01 and VI-02 stained distinctly kidney glomerulus and in sections of liver tissue VI-01, VI-03 and VI-05 antibodies strongly stained sinuses. In sections of testes, VI-01, VI-02, VI-03 and VI-04 antibodies stained Leydig´s cells. All 5 antibodies exhibit positive reactions with Sertoli´s cells and only VI-03 antibody stained spermatozoa.

459 VERTEBRATE LIVER CYTOKERATINS. A COMPARATIVE STUDY.
R. G. Pankov, A. A. Uschewa, G. G. Markov. Institute of Molecular Biology, Bulgarian Academy of Sciences, Sofia 1113, Bulgaria

The intermediate filaments /IF/ and their proteins in livers from representatives of different vertebrate classes / Pisces, Amphibia, Reptilia, Aves and Mammalia/ have been studied by electron microscopical, biochemical and immunochemical methods. In all cases the IF display an equal electron microscopic morphology. The molecular weight of the IF-proteins varies from 40000 to 60000 and their pI - between 5.0 to 6.45. Immunological investigations show that in all animals studied the IF are built up of cytokeratins belonging to the two types of keratins - type I and type II. In the course of vertebrate evolution, the pI of type II cytokeratins has become more alkaline. The acidic type I cytokeratins also tend to increase the pI although in s shorter range. As a result during vertebrate evolution, the difference of pI between cytokeratins of the two types has been significantly increased. In all cases only one protein of type II cytokeratins is present while the cytokeratins of type I are represented by a single protein only in the liver IF of the higher vertebrates /mammals/. In lower vertebrates two or even three type I cytokeratins contribute to the structure of the IF. The presence of unpaired type I keratins put the interesting question of the organization of the intermediate filaments in such cases.

460 THE BEHAVIOUR OF CYTOKERATINS IN HUMAN ORAL PRENEOPLASIAS AND CARCINOMAS
J. Schulz (1), T. Ermich (2), D. Schumann (2), G. Raabe (3). (1) Institute of Biochemistry, Humboldt-University, DDR-1040 Berlin, Hessische Str. 3-4, (2) Hospital of Maxillo-facial and Plastic Surgery and (3) Institute of Pathology, Friedrich-Schiller-University, DDR-6900 Jena, Bachstr. 18. G.D.R.

Human oral tissues were prepared and extracted as described by Franke et al.(J. Mol. Biol. 183 (1981) 933) and analyzed for the pattern of cytokeratins (PCK) by means of SDS-PAGE. The non-cornified mucous membrane contains polypeptides with 59, 55, 52.5, 50, and somtimes 48 and 45 kD. They correspond to the cytokeratins (CK's) No. 4, 12, 8, 14/15, 16, and 18, resp., of the CK catalog of Moll et al. (Cell 31 (1982) 11). The keratinized gingiva possesses the same CK's and additionally No. 1 (68 kD) and 3 (63 kD). Under normal healthy conditions the PCK is relatively stable at each site and very similar from day to day and from person to person.
In preneoplasias such as leukoplakia and lichen ruber and in squamous carcinomas of oral epithelium the PCK deviated more or less from the normal pattern. Often the CK's No. 1 and 3 appeared irregularly regardless wether cornification took place or not. Moreover, the M_r value of few polypeptides (67, 61, 49, 44, 42 kD) differed from the typical M_r of CK's but could be produced from them by partial proteolysis. The variability of PCK was most pronounced in the preneoplasias studied and were in striking contrast to the only quantitative changes of CK's in cases of gingival inflammation and hyperplasia.
The results confirm the thesis that under pathologic conditions the PCK may (but must not) be changed by switching the expression of the multiple CK genes and the processing of their products, the regulatory mechanisms of which are still unknown.

461 TUMOR-PROMOTER-INDUCED DISRUPTION OF JUNCTIONAL COMPLEXES IN EPITHELIAL CELLS IS FOLLOWED BY THE INHIBITION OF CYTOKERATIN AND DESMOPLAKIN SYNTHESIS
Avri Ben-Ze'ev, Department of Genetics, Weizmann Institute of Science, Rehovot 76100, Israel

The organization and synthesis of proteins involved in the formation and the stabilization of desmosomes was investigated in epithelial cells treated with TPA. In kidney MDBK and MDCK epithelial cell colonies TPA induced a rapid disruption of desmosomes and marked alterations in cell morphology. These changes in cell- and colony-morphology were followed by a coordinated inhibition in the synthesis of all cytokeratins and a high M.W. desmoplakin (desmoplakin I) in MDBK and MDCK cells after 24 hr of TPA treatment, but without affecting vimentin synthesis which is coexpressed in these cells. In BMGE cells which were derived from the bovine mammary gland, TPA inhibits especially a 45 kd acidic cytokeratin which was previously shown to be synthesized at high levels only in dense cell cultures (A. Ben-Ze'ev, Exp. Cell Res. 157, 520, 1985). The inhibition of cytokeratins synthesis in TPA treated cells was reflected also at the level of mRNA translatibility in vitro. In very dense cell cultures, TPA had only minor effects on cell morphology, the desmosomal junctions were still maintained, and the level of cytokeratins synthesis was not affected. The extent of cytokeratins phosphorylation was also found to be unaltered by TPA treatment in either isolated colonies or in dense cell cultures. These results support the existence of a linkage between the regulation of synthesis and the organization of cytoskeletal proteins which are involved in desmosomes formation (A. Ben-Ze'ev, J.C.B. 99, 1424, 1984), and an independent regulation of the synthesis and phosphorylation of intermediate filament proteins (A. Ben-Ze'ev, J.C.B. 97, 858, 1983).

462 ALPHA-KERATINS OF HUMAN AND ANIMAL HAIR: A SPECIFIC SUBSET OF EIGHT POLYPEPTIDES DIFFERENT FROM EPITHELIAL CYTOKERATINS.

Hans Heid, Ekkehard Werner, Werner W. Franke. Institute of Cell and Tumor Biology, German Cancer Research Center, Im Neuenheimer Feld 280, D-6900 Heidelberg, FRG

A method has been developed for the preparation of living hair root (matrix) cells from different species (human, bovine, sheep) without the inner and outer root sheath cells. The major cytoskeletal proteins found were intermediate filament (IF) polypeptides, corresponding to the "low sulfur keratin" fraction of mature hair and sheep wool. Using immunological and protein chemical criteria as well as IF reconstitution experiments, these polypeptides can be divided into an acidic (type I) subfamily of M_r 40,000-43,000 and a basic (type II) subfamily of M_r 56,000-59,000 which are remarkably similar in the various species and hair types. These polypeptides are related to - but not identical with - the identified cytokeratins of epithelia (Moll et al., Cell 31, 11-24, 1982). In vitro translation assays using mRNA isolated from hair follicles shows that these polypeptides are genuine products of discrete mRNAs, suggesting that hair formation involves the switching off of genes encoding epithelial cytokeratins and the induction of expression of a specific subset of cytokeratin genes.

463 TRIDIMENSIONAL ORGANIZATION OF ACTIN MICROFILAMENTS IN THE APICAL BORDER OF OVIDUCT CILIATED CELLS.

G. Nicolas(1), B. Chailley, M.C. Lainé(2). (1) Laboratoire de Cytologie, Université Pierre et Marie Curie, Laboratoire de Technologie CNRS, 105 Bd Raspail, 75272 Paris, France, and (2) Centre de Biologie Cellulaire CNRS, 67 rue Maurice Günsbourg, 94200 Ivry sur Seine, France.

The polarized epithelial cells have a well-developed apical cytoskeleton which is well known in enterocytes. In ciliated cells from Vertebrates, few data documents the cytoskeleton network. The comparative study by thin sectioning and freeze fracture-deep etching allows to understand a tridimensional organization of the cytoskeleton. The cortical cytoplasm is mainly occupied by the numerous basal bodies and their accessory structures, the lateral basal foot and the striated rootlets. Intermediate filaments form a subterminal meshwork related to the striated rootlets.

After S_1 fragment decoration, the distribution of actin microfilaments have been revealed in the apical cytoplasm of ciliated cells from quail oviduct. Microfilament bundles coming from microvilli are interspersed between the basal bodies. Some microfilaments are closely associated with the basal foot tip forming a slight network between the kinetosomes. This network is denser at the proximal part of kinetosomes, and it is related to an annulus of actin which is associated to the junctional zonula adhaerens. The anchoring fibers located at the distal pole of kinetosomes are associated with microfilaments which are running around the whole kinetosome perpendicularly to the cell surface. Using indirect immuno gold-labelling, actin was found in the same localization. The grid pattern organization of actin described in ciliated cells of mussel gills (1) was not found in quail oviduct.

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464 A NOVEL MICROFILAMENT PROTEIN WITH MOLECULAR WEIGHT 53000 DALTON DETECTED BY MONOCLONAL ANTIBODY.

K.I.Galaktionov, I.I.Frydlanskaya, D.B.Gromov, G.P.Pinaev. Institute of Cytology, Academy of Sciences of the USSR, 194064 Leningrad, Tichoretsky av., 4.

Several hybridomas producing monoclonal antibodies (Mab) to microfilament proteins were obtained from mice injected with crude cytoskeleton of 3T3 cells. One of these Mab's - E9-48 (IgM,k) stained bundles of microfilaments intensively and permanently through their full length. According to immunoblotting analysis this Mab revealed a protein with molecular weight 53000 dalton (53 KD) in 3T3, HeLa, mouse embryonal fibroblasts (MEF) and other mammalian cells. In addition, protein with the same molecular weight was detected in myofibrills from rabbit psoas muscle. Monoclonal antibody E9-48 showed intensive staining of microfilament network in the leading edge of MEF and surface microspikes and microvilli in HeLa and A-431 cells. Double immunofluorescence studies with Mab E9-48 and anti-actin antibodies showed the similar distribution of actin and 53 KD in mammalian cells (A-431, NRK, 3T3, etc). Antibody binding sites on microfilaments were visualized on electronmicroscopic level by immunogold staining technique. Protein under investigation was found to be localized permanently through the length of actin bundles and sometimes as a small clusters. The coincidence of 53 KD and actin distribution in 3T3 and A-431 cells did not change after treatment by cytochalasin B and EGF. Colcemid had no significant effect on 53 KD distribution within microfilaments of these cells. Monoclonal antibodies E9-48 did not crossreact with fascin from sea urchin eggs. These results lead us to the suggestion that 53 KD is a novel microfilament protein in mammalian cultured cells.

465 APPEARANCE AND ARRANGEMENT OF FILAMENTOUS PROTEINS IN HUMAN TUMORS

Heinz DAVID and Ingrid UERLINGS. Department of Ultrastructural Pathology and Electron Microscopy, Institute of Pathology, Charité Clinic, Humboldt University Berlin, GDR.

By immunohistochemical investigations today it is possible to demonstrate especially the intermediate filaments (e.g. cytokeratins, vimentin, desmin, acidic glial filaments, neurofilaments) in tumor cells. By the immunohistochemical method tumor cells are characterizeable in regard to the genesis and development from epithelial and mesenchymal tissue. The evidence depends among other things on the amount of the filaments in the cell.

Electron microscopically the characterization of the different filaments is impossible. Nevertheless the arrangement of the filaments in the cells, relationships between the filaments and the organelles and the plasma membrane, and also the arrangement in single forms or in bundles are important. These findings are demonstrated in several types of carcinomas, sarcomas and brain tumors.

466 EFFECT OF DIMETHYL SULFOXIDE (DMSO) ON THE CYTOSKELET OF TUMOUR CELLS.

Jana Čapková, Jana Pěknicová. Institute of Molecular Genetics, Czechoslovak Academy of Sciences, 142 20 Praha 4, Vídeňská 1083, Czechoslovakia

The mechanism of action of the cryoprotective agent DMSO on the cytoskelet of tumour cells was tested in mouse fibroblasts of the MEF-MCH line. After treatment with DMSO (15%DMSO in culture medium for 10 min) the cells were extracted with 0.15% Triton X-100 and the cytoskeleton component - microtubules - were visualized using TU-01 monoclonal antibody against tubulin. In DMSO-treated cells, unlike DMSO-untreated cells, it was impossible to demonstrate microtubules with the anti-tubulin antibody. The DMSO-induced depolymerization of microtubules was reversible, and a 4-hr incubation of DMSO-treated fibroblasts in culture medium without DMSO led to complete reconstruction of microtubules together with restoration of cell shape.

467 THE ROLE OF MYOSIN IN LOCOMOTION AS STUDIED BY MICROINJECTION

B.Höner, Chr. Wiegand and B.M. Jockusch
Developmental Biology Unit, University of Bielefeld, 4800 Bielefeld, FRG

Locomotory activity of tissue culture cells is thought to depend on cytoplasmic microfilaments. Structural analysis of the leading edge of migrating cells has revealed actin filaments as part of a cortical web, presumably responsible for locomotion (1,2). However, until now, direct evidence for participation of myosin in locomotion is missing. We attempted to investigate such a presumptive role of myosin by microinjecting a specific antibody against smooth muscle myosin into locomoting cells. This antibody, a polyclonal IgG, affinity purified on nitrocellulose, crossreacts with nonmuscle myosin, as seen in immunofluorescence and in immunoblots. When microinjected into stationary epithelial cells and fibroblasts, this antibody selectively precipitates myosin from stress fibers (3,4). Therefore, this antibody seemed a useful tool to study the effect of myosin depriviation on the locomotory activity of migrating cells. The results of such an analysis will be discussed.

Supported by the Deutsche Forschungsgemeinschaft, the Stiftung Volkswagenwerk and the ASTA/Degussa Pharma Group.

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468 PURIFICATION AND CHARACTERIZATION OF MYOSIN FROM EHRLICH ASCITES TUMOR CELLS

Jacek M. Kuźnicki, Anna Kowalczyk. Nencki Institute of Experimental Biology, 02-093 Warsaw, 3 Pasteur, Poland

Myosin from Ehrlich ascites tumor cells was purified to homogeneity. The purification procedure included extraction with a low ionic strength buffer, DE-cellulose column chromatography at pH 8.0, and precipitation of myosin filaments by dialysis against buffer containing no salt. A precipitate consisted of myosin, a small amount of a 90 kDa protein and actin. The impurities were separated from myosin during gel filtration on a Sepharose 4B column. The yield was about 8 mg from 100 g of cells. Myosin from Ehrlich ascites tumor cells consists of the heavy chains of about 200 kDa and of two classes of light chains, 20 kDa and 16 kDa. The myosin exhibits much higher Ca^{2+}- ATPase activity /0.3 μmolexmin^{-1}xmg protein^{-1}/ than K^{+}/EDTA ATPase activity. Its Mg^{2+}- ATPase activity is low and it is practically not activated by F-actin under conditions used. Incubation of the cells in Hanks' medium supplemented with /^{32}P/-ortophosphate and subsequent isolation of myosin revealed that the 20 kDa light chains are phosphorylated in vivo. Phosphorylation of the same light chains was also observed in vitro with the use of myosin light chain kinase from chicken gizzard. The effect of the light chain phosphorylation on the actin-activated ATPase activity of myosin from Ehrlich ascites tumor cells is presently under investigation.

469 SIGNIFICANCE OF THE SH/SS-STATUS OF CYTOSKELETAL PROTEINS IN HUMAN BLOOD PLATELETS

P. Spangenberg (1), Regine Heller (1), Amalia Bosia (2), P. Arese (2) and U. Till (1) (1) Institute of Pathological Biochemistry, Medical Academy of Erfurt, 5010 Erfurt, GDR and (2) Department of Chemistry and Propedeutical Biochemistry, Institute of Hygiene, University of Torino, 10126 Torino, Italy.

The cytoskeleton of blood platelets is composed of microfilaments and microtubules, intermediate filaments have not been detected yet. The maintenance of the sulfhydryl/disulfide-status of microfilamental proteins is essential for a normal platelet reactivity. Oxidation of intact cells by diamide (azodicarboxylic acid-bis-dimethylamide) leads to the formation of disulfide-linked polymers with different molecular weights composed of certain cytoskeletal proteins. The polymer formation is dynamical. Formed polymers can be reduced by the repair mechanism of the cell (GSH/GSSG-system) or by incubation of diamide-treated cells with 2-mercaptopropionylglycine. The polymerization influences the reactivity of the platelets. The appearance of a double band of the polymer a (P_a), which is a dimer of myosin, correlates with reversible aggregation of otherwise irreversibly aggregating platelets. The formation of a high molecular weight polymer (P_c), which is composed of different microfilamental proteins, is always accompanied by a complete inhibition of aggregation. The reduction of P_c results in a normalization of the aggregation behaviour. The organization of phosphatidylethanolamine (PE) within the plasma membrane of the cell is not influenced by oxidation of certain cytoskeletal proteins, whereas the thrombin/collagen-induced PE-translocation is markedly inhibited by diamide. Modification of the SH/SS-status of cytoskeletal proteins permits the investigation of structure and function of the cytoskeleton within platelets.

470 TROPOMYOSIN CO-LOCALIZES WITH MICROFILAMENTS AND MICROTUBULES IN INNER EAR SUPPORTING CELLS

Norma Slepecky and Steven C. Chamberlain. Institute for Sensory Research, Syracuse University, Syracuse NY USA and Depts. of Anatomy and Cell Biology, SUNY Upstate Medical Center, Syracuse NY USA.

S-1 decoration has previously shown that actin is localized in the tunnel pillar cells and the Deiter's cells within the organ of Corti. In the reticular lamina and at the basilar membrane actin filaments are organized as a meshwork of randomly oriented filaments. In the processes of the supporting cells, holding the organ of Corti up off the basilar membrane, actin is present as filaments interdigitating with microtubules.

A similar pattern for the distribution of actin in supporting cells was observed with immunofluorescence. Antibodies to tubulin are localized in the region of the supporting cells holding the organ of Corti up off the basilar membrane and extending around to the apical surface of the reticular lamina. Ultrastructurally, in all these places microfilaments and microtubules have been observed.

Antibodies to tropomyosin co-localize with antibodies to tubulin and with the distribution of actin microfilaments where they interdigitate with microtubules. The role of tropomyosin in conferring structural rigidity to the actin filaments is discussed.

471 STUDY OF MICROTUBULES AND MICROFILAMENTS NETWORKS DURING VOLUME REGULATION IN CULTURED PC12 CELLS.

M.Cornet, E.Delpire, C.Duchêne and R.Gilles
Laboratory of Animal Physiology, University of Liège, 22, Quai Van Beneden, Belgique.

Cultured rat pheochromocytoma cells of line PC12 show, as most other cell types, a volume regulation process when submitted to a hypoosmotic shock (NaCl/2)(Delpire et al., 1985). The eventual role of microtubules and microfilaments in this response has been studied by light microscopy following ImmunoGold/Silver Staining. The obtained data show that the general configuration and density of the microtubular system is not significantly different in cells submitted to a hypoosmotic medium (NaCl/2) or maintained in an isosmotic one. On the other hand, the microfilamentar lattice shows important changes in configuration and density in cells submitted to a hypoosmotic shock for at least 20 minutes. These modifications are similar to those seen in cells traited with cytochalasin B (0,05 mM) in isosmotic conditions. These results, together with data on cell volume and ions content obtained in the same conditions, are discussed in the framework of current concepts in cell volume regulation.

Reference

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Effects of osmotic shocks on the ion content and ultrastructure of rat pheochromocytoma cells of line PC12. Mol. Physiol., 8 : 293-306.

472 FILAMENTOUS MATRIX OF SUBPLASMALEMMAL CALCIUM-BINDING MICROREGIONS IN ACANTHAMOEBA

A.Sobota, Nencki Institute of Experimental Biology, 02-093 Warsaw, Poland

Electron microscopy studies of subplasmalemmal layer of Acanthamoeba showed the presence of calcium/phosphorus-containing, electron-dense structures (Ca-DS). The diameter of the structures was ca. 70 nm in the cells fixed with glutaraldehyde-Ca, and their frequency was 3.7 Ca-DS/µm membrane. Analysis of thin-sections of Epon-embedded specimens detected that Ca-DS were closely associated with the microfilament meshwork, which seemed to be inserted in the electron-dense material of Ca-DS. Mild removing of the electron-dense material from Ca-DS displayed fine, well-organized filamentous matrix inside "the empty" Ca-DS. The matrix was composed of three hemispheres of different radii (ca. 7, 20, 35 nm) inserted one into another and associated with cell membrane. The hemispheres were also radially cross-linked by 2 nm filaments, which were also attached to the plasma membrane.

Appearance of a filamentous substructure in "the empty" Ca-DS as well as the association of Ca-DS with the microfilament meshwork suggested that in the presence of a high level of calcium ions the aggregation of some cytoskeletal/membrane components occurred. The formed subplasmalemmal aggregates served as a matrix for the binding of Ca/P salts and thus for the forming of the electron-dense structures.

473 CYTOCHALASIN RESISTANT MUTANTS OF FRIEND ERYTHROLEUKAEMIA CELLS. Ivor Hickey(1), Rosemary Neill(1), & Anne Hughes(2). (1) Department of Genetics and (2) Department of Medical Genetics, The Queen's University, Belfast, Northern Ireland

Spontaneous mutants resistant to Cytochalasins B and D were isolated from the Friend erythroleukaemia cell line. These mutants arose at a rate of 6.8×10^{-8} per cell generation. The mutants were cross-resistant to cytochalasin D and showed no decreased sensitivity to the inhibition of deoxyglucose uptake by cytochalasin B. Thus suggesting that the mutation affects the cytoskeleton of the cells rather than membrane transport mechanisms. 2-D gel electrophoresis was carried out to compare the actin polypeptides of parental and resistant cells. No differences were noted in the migration α and β actins from the two cell types. The distribution of actin as detected by indirect immunofluorescence was the same in both cell types before and after exposure to CB.

Hybrids were constructed between cytochalasin resistant mutants and parental cells. In 11 of 12 independent hybrid clones the resistant phenotype was found to be fully dominant. However in a series of hybrids with mouse lymphoma cells and Chinese hamster fibroblasts all hybrids were found to have the same phenotype as the sensitive parent.

The molecular alteration within the cell responsible for cytochalasin resistance is at present not known. However the behaviour of the resistant phenotype in hybrids suggests that it may be associated with erythroid-specific elements of the cytoskeleton in Friend erythroleukaemia cells.

474 PROTEINS OF THE ERYTHROCYTE SKELETON IN FRIEND LEUKEMIA CELLS.

M. Ceccarini, P. De Cesaris, M. Minetti, S. Barca. Cell Biology Dept., Istituto Superiore di Sanità, Viale Regina Elena, 299 - 00161 Rome, Italy.

In the present study we have investigated the presence of red blood cell (RBC) cytoskeletal proteins in Friend erythroleukemia cells (FLC) and their modifications during dimethylsulfoxide (DMSO) induced cell differentiation. By using immunochemical techniques (immunofluorescence, immunoprecipitation, immunoblotting) we have detected the presence of spectrin and protein 4.1 in FLC extracts. Furthermore, both these proteins accumulate several times after DMSO-induced cell differentiation and are preferentially located in the cytoskeletal-TRITON-X-100-insoluble fraction. At present the structural organization of these proteins in FLC is unknown. In mature RBC most of the cytoskeletal proteins including the β subunit of spectrin are phosphorylated and, significantly, the phosphorylation sites are in the same proteolytic domains of the binding sites for other cytoskeletal proteins. A role of phosphorylation in the cytoskeleton assembly of RBC has been proposed. In order to investigate a possible role of phosphorylation in the FLC cytoskeleton organization, we labeled FLC with ^{32}P-orthophosphoric acid, incubating the cells at 37°C for a period of time from 1 to 16 hours. In these conditions none of phosphorylated bands showed the electrophoretic mobility of spectrin β subunit. At the same time, no phosphorylated band was immunoprecipitated by using polyclonal antispectrin antibodies. Our preliminary results indicate that spectrin seems non-phosphorylated in FLC at variance of mature RBC.

This work was partially supported by a grant of Italian National Research Council, Special Project "Oncology" contract n. 85.02560.44.

475 CYTOSKELETAL PROTEINS IN NICOTIANA POLLEN TUBE

Antonio TIEZZI and Mauro CRESTI

Dept. of Environmental Biology - Botany Section, Via Mattioli 4, 53100 SIENA, ITALY.

Pollen tube growth is related to the presence of cytoskeletal proteins that are involved in cytoplasmic streaming, generative cell movement and transport of cell wall precursors.

Nicotiana pollen grains were grown in vitro and the presence of actin and tubulin was evidentiated by a two dimensional gel electrophoresis. The distribution of microfilaments was visualized by rhodamine - conjiugated phalloidin. Microtubules arrays was investigated by an antibody against tubulin. Electron microscopical observations allowed to localize microtubules in generative cells.

Both types of filaments are oriented along the longitudinal axis of the pollen tube and their arrangement seem to be similar. Not identified proteins are likely to form connections beetwen microfilaments and microtubules.

476 CHEMOTACTIC FACTOR(S) IN MOUSE KIDNEY CONDITIONED MEDIUM FOR METASTATIC MOUSE LYMPHOMA CELLS.

Rosemarie Benke and Volker Schirrmacher, Institut für Immunologie und Genetik, Deutsches Krebsforschungszentrum, D-6900 Heidelberg, FRG

A plastic adherent variant cell line (ESbMP) generated from a high metastatic non adherent mouse lymphoma (ESb) was found to metastasize nearly always in the kidney (80% of the mice), whereas only very few kidneys were infiltrated by ESb cells (8% of the mice).

To test if the kidney produced any attracting factors for the ESbMP cells which might cause this high level of kidney metastases we cultured kidney of normal mice, collected kidney conditioned medium (KCM) and tested it for chemotactic and other activities on the tumor cells in vitro.

While we could not find any mitogenic effect of KCM for the tumor cells, stimulated migration was seen when using a modified Boyden chamber assay. Migration was significantly enhanced only when KCM was placed in the lower chamber. This means that KCM stimulated directed migration, and exerted thus a true chemotactic effect. A chemokinetic effect - stimulation of random locomotion -, however, could not be detected. ESb cells responded much less to KCM than ESbMP cells.

Recently we succeeded in serum-free preparations of the KCM. This showed the same effects as the KCM described above, so that further biochemical analysis is facilitated.

In serum-free KCM the motility effects were shown to increase with preincubation of KCM at 37°C for two to four hours. After 24 h preincubation, however, the activity disappeared completely. The factor(s) appeared to be heat stable (30' at 56°C) and to disappear after dialysis thus indicating low molecular weight.

477 INHIBITION OF NEUTROPHIL CHEMOTAXIS AND METABOLIC BURST BY ANTAGONISTS OF INTRACELLULAR CALCIUM.

Jan G.R. Elferink and Martha Deierkauf. Dept. Medical Biochemistry, Sylvius Labs, University of Leiden, POB 9503, 2300 RA Leiden, The Netherlands.

Under some experimental conditions rabbit neutrophils may perform chemotaxis or a metabolic burst in the absence of extracellular Ca^{2+}. This enables the study about the role of intracellular Ca^{2+}, using suitable antagonists. As anatagonists TMB8, quin2 (presented to the cells as quin2-AM), chlortetracycline, chlorpromazine and prenylamine were used; all these agents are known to interfere with Ca^{2+}. The effect of these antagonists, and the modulation of the effect by extracellular Ca^{2+}, on chemotaxis towards f-Met-Leu-Phe was compared with the effect on the metabolic burst. The effect on activation of the metabolic burst by f-Met-Leu-Phe (+cytochalasin B) was compared with that on activation by phorbol myristate acetate (PMA). Neutrophils were isolated from the rabbit peritoneal cavity. The metabolic burst was measured as nitrobluetetrazolium dye reduction. All antagnosits inhibited both chemotaxis and the metabolic burst in the absence of extracellular Ca^{2+}. The inhibition of all agents on chemotaxis, and the effect of TMB8, quin2 and chlortetracycline on the metabolic burst, was antagonized by extracellular Ca^{2+}. The effect of these agents is thus clearly Ca^{2+}-antagonistic. Inhibition of chemotaxis was different from that of the metabolic burst: TMB8, chlorpromazine and prenylamine gave less inhibition of chemotaxis than of the metabolic burst, irrespective of the kind of activator. There are also differences between inhibition of f-Met-Leu-Phe activaton and PMA-activation of the metabolic burst: TMB8 and especially chlortetracycline inhibit PMA activation more effectively than f-Met-Leu-Phe activation. The results are consistent with the view that intracellular Ca^{2+} is required in chemotaxis, and in PMA or f-Met-Leu-Phe activated metabolic burst, but that the role of Ca^{2+}, or the location where Ca^{2+} is derived from, is different and depends on functior and the type of activator.

478 CYTOSKELETAL ARCHITECTURE AND SUBCELLULAR LOCALIZATION OF CONTRACTILE PROTEINS IN CULTURED CARDIOMYOCYTES BY PLATINUM REPLICA IMMUNOELECTRON MICROSCOPY. Yuji Isobe and Larry F. Lemanski. Department of Anatomy and Cell Biology, State University of New York, Health Science Center at Syracuse, Syracuse, New York 13210, U.S.A.

Cardiac myocytes obtained from neonatal hamsters were cultured on carbon-coated glass coverslips for 2-3 days. They were physically permeabilized by breaking open the cell membranes to expose the internal cytoskeletal networks. The method of physical rupture of cell membranes was as follows: poly-L-lysine coated clean coverslips were placed on top of the cells and then peeled apart from the original coverslips within the buffer (70 mM KCl, 5 mM $MgCl_2$, 3 mM EGTA, 30 mM HEPES, 10 µM Taxol, 0.25 mM PMSF; pH 7.0). Samples were lightly fixed with paraformaldehyde and treated with specific primary antibodies, then further indirectly immunolabelled with colloidal gold particles. After sequential fixation steps in glutaraldehyde, tannic acid, and osmium tetroxide, samples were immersed in 70% ethanol and frozen by plunging them into Freon 13 cooled by liquid nitrogen. The cells were freeze-dried for 30-60 minutes and rotary-replicated with platinum and carbon in a freeze-fracture apparatus. This rotary replica method on physically-opened cells enabled us to view the spatial organization of cytoskeletal as well as membranous structures. On the inside surface of the plasma membranes, many 8-10 nm filaments together with finer 2-4 nm filaments had formed complex networks. Clathrin coats were well preserved and showed a unique honeycomb-like appearance. Nascent myofibrils were seen as densely-packed filament bundles within the deeper cytoplasm. When the immuno-gold technique was combined with this method, the high resolution subcellular localization of specific protein molecules was clearly demonstrated in three dimensions. (Supported by NIH Grant HL32184 and a Grant-in-Aid from the American Heart Association.)

479 PHOTODYNAMIC EFFECTS OF BONELLIN ON MUSCLE CONTRACTION

Marina De Nicola Giudici, Dept. Genetics, General & Molecular Biology, University of Naples, V. Mezzocannone, 8 Napoli, Italy.

Bonellin, the pigment of the marine worm Bonellia viridis, is a photosensitizer highly toxic against prokaryotic and eukaryotic organisms, blocking cellular division, inhibiting cell adhesion and embryonic development. Since bonellin blocks also irreversibly the motility, it has been studied his mechanism of action in vivo on Ciona larvae and in vitro on isolated myofibrils of frog muscle. 19hr aged larvae were treated with 5 µg/ml bonellin under 10 min photoirradiation and isolated myofibrils, treated with 5 µg/ml bonellin, were photoirradiated for 1 hr. The contractile proteins were extracted and submitted to electrophoretic analysis. Samples were prepared for EM.

The electophoretic analysis shows that bonellin induce strong cross-linking in myosins chain (HC, 200K), paramyosin (PM, 95K), myosin light chain (LC, 18K), while actin is unaffected. This effect is observed in insoluble matter after bonellin treatment.

Corrispondently EM observations indicate that bonellin effect results in enlargement of fibrils and erosions areas were observed, as consequence of alteration of thick filaments. It has been concluded that the inhibition of motility caused by bonellin is due to cross-link of myosins.

480 CHARACTERISTIC CONTRACTILE FEATURES OF THE CARDIAC VALVULAR INTERSTITIAL CELLS.
Doina A.Filip, Aurelian Radu and Maya Simionescu, Institute of Cellular Biology and Pathology, Bucharest - 79691, Romania

Interstitial cells of heart atrioventricular and sigmoid valves were examined in several laboratory animals (rabbit, hamster, rat and mouse) and in humans. These cells constitute a large fraction of the total cell population of the valve; in mouse atrioventricular valve, they amount to 30% of volumetric density (estimated by point-count volumetry). By their ultrastructural features and functional properties, valvular interstitial cells (VIC) are intermediate between fibroblasts and vascular smooth muscle cells. VIC lack basal lamina establishing direct and extensive contacts with collagen fibres, elastin microfibrils and proteoglycans of the matrix. The cells have numerous slender and long processes, connected to one another, forming a complex cellular framework spanning the entire valve. Motor nerve endings are located closely apposed to VIC; structurally most of them appear to be of adrenergic type. As shown by thin sections, freeze-fracture, lanthanum staining and carboxyfluorescein microinjection, VIC are extensively coupled by communicating junctions. These cells contain numerous bundles of actin filaments, as demonstrated by decoration with the S_1 fragment of heavy meromyosin. VIC also express cyclic GMP-dependent protein kinase, as detected by immunoflorescence and immunoperoxidase histochemistry. These results reveal that VIC possess the general machinery necessary for contraction whose presence may account for a controlled tonus actively correlated with the cyclically changing forces acting on valves during diastole and systole. (Supported by Ministry of Education, Romania, and by NIH Grant HL-26343).

481 CONTRACTILE AND CYTOSKELETAL DOMAINS IN VERTEBRATE SMOOTH MUSCLE:
J.V. Small[+], D.O. Fürst[+], R.A. Cross[+], H. Hinssen[+] and J. De Mey[*]. [+]Institute of Molecular Biology, Austrian Academy of Sciences, Billrothstr. 11, 5020 Salzburg, Austria, and *Janssen Pharmaceutica, Beerse, Belgium.

Recent immunocytochemical analysis has revealed the existence of two distinct fibrillar domains in smooth muscle cells: contractile domains containing actin and myosin and "cytoskeletal" domains containing intermediate filaments, dense bodies and actin. In the actomyosin domains no periodic distribution of actin or myosin label could be detected in the longitudinal direction in the light microscope, indicating a continuous distribution of actin and myosin filaments within these fibrils. For myosin, this feature was particularly evident in isolated cells whose structure was loosened by partial removal of actin using an actin-fragmenting protein purified from smooth muscle.

Two actin-binding proteins that occur in considerable amounts in smooth muscle, caldesmon and filamin, are spatially segregated from each other in the two separate domains: caldesmon in the actomyosin domains and filamin in the cytoskeletal domains. Both proteins show an extended and flexible molecular morphology, are dimeric and are capable of cross-linking actin filaments into gelled arrays. It is proposed that such cross-linking activity contributes to tension maintenance, or tone, in vertebrate smooth muscle.

482 MEMBRANE - CYTOSKELETON INTERACTIONS DURING SPREADING AND LOCOMOTION OF CULTURED CELLS.
J.M.Vasiliev, Cancer Research Center of the Academy of Medical Sciences, 24 Kashirskoje Shosse, Moscow and Moscow State University, Leninskije Gory, Moscow, USSR.

Mechanisms of pseudopod extension, retraction and attachment in cultured fibroblasts and epithelial cells will be discussed. The actin network formed in the pseudopod is suggested to be a primary structure, from which other actin structures are formed. Centripetal movement of this network is, possibly, involved in many membrane-controlled phenomena, e.g. in the assembly of matrix fibrils or formation of cell-substrate contacts. Cell-cell contacts can interrupt the centripetal movement of pseudopods; the role of this interruption in the contact inhibition of movements will be discussed. Extension and retraction of pseudopods are also controlled by interrelationships of actin cortex with microtubular system; alterations of these interrelationships caused by tumor promotor, TPA, will be described.

483 REVERSIBLE INTERACTIONS BETWEEN α-ACTININ, VINCULIN AND LIPID MODELS, AS WELL AS WITH THE CELL MEMBRANE. Verena Niggli, Paul Burn and Max M. Burger, Dept. of Biochemistry, Biocenter of the University of Basel, Klingelbergstr. 70, CH-4056 Basel, Switzerland.

The cytoskeletal proteins vinculin and α-actinin have been implicated to mediate the interaction of microfilaments with plasma membranes. Very little direct evidence for this hypothesis has yet been put forward, and the interaction of these proteins with membranes (be it with transmembrane protein receptors or lipids) has not been extensively studied. Recently it has been shown that α-actinin forms a highly specific stoichiometric complex with diacylglycerol and fatty acids *in vitro* (1). Moreover, diacylglycerol and palmitic acid appear to functionally regulate α-actinin, as they induce *in vitro* the formation of large aggregates of α-actinin and actin of the type observed in actin filament bundles (2). Furthermore, such α-actinin/lipid complexes can also be formed *in situ* in stimulated blood platelets. For instance, cytoskeletons isolated from activated platelets contain at least 30 fold more diacylglycerol and palmitic acid, compared to cytoskeletons from resting platelets. α-Actinin, immunoprecipitated from cytoskeletons of activated platelets, contains very tightly bound diacylglycerol (2). It is not yet known how these lipids interact with α-actinin on a molecular basis, and how they are involved in the cellular reorganizations of the cytoskeleton. Thrombin-induced activation of platelets leads to a rapid production of diacylglycerol, and at the same time to actin polymerization and reorganization of the cytoskeleton. These two events may be connected, as a complex of diacylglycerol, fatty acid and α-actinin can initiate bundling of actin filaments in membranes. As none of the components involved is unique to platelets, this mechanism could be general in regulating cytoskeleton/membrane interaction during cell-shape changes and migration.

We have now obtained evidence that also vinculin, considered until now to be a cytosolic, soluble protein, can interact, at least *in vitro*, reversibly with membranes. To investigate a possible direct interaction of vinculin with bilayers, photolabeling with a phospholipid generating a highly reactive carbene was used (3). This phosphatidylcholine analogue, 1-palmitoyl-2 [-10-[4-[(trifluoromethyl) diazirinyl] phenyl]-[^{3}H]9-oxaundecanoyl]-sn-glycero-3phosphocholine ([^{3}H]PTPC/8), with the photoactivatable diazirine group on its apolar portion, has been shown to label selectively membrane-embedded domains of proteins. Vinculin was incubated and photolyzed with liposomes containing 1% (w/w) of this photoactivatable phospholipid, followed by chloroform/methanol extraction to remove non-covalently bound lipid, and finally SDS-gel electrophoresis. The 130'000 dalton band of vinculin is significantly labeled after this procedure, but only when the liposomes also contain acidic phospholipids such as phosphatidylinositol or phosphatidylserine. All acidic phospholipids tested so far increase markedly (5-17-fold) labeling of vinculin, compared to labeling in neutral phospholipids such as phosphatidylcholine. Labeling is maximal at low ionic strength, but significant vinculin labeling can still be observed at physiological salt concentrations and acidic phospholipid content of the membrane. Our data suggest strongly that vinculin inserts into the hydrophobic part of the bilayer by interacting with acidic phospholipids. In addition an interesting effect specific for phosphatidylinositol was observed: Interaction of vinculin with phosphatidylinositol liposomes induces a shift of the apparent molecular weight of the protein in SDS-gels to slightly higher values (135'000 dalton instead of 130'000 dalton). This effect is independent of the presence of [^{3}H]PTPC/8 and is only induced by phosphatidylinositol, but not by other acidic phospholipids, such as phosphatidylserine. Vinculin, when incubated with ^{14}C-phosphatidylinositol, is clearly labeled, after SDS gel electrophoresis, implicating the formation of very tight protein-lipid complexes, that are even stable in SDS. We are now studying the exact nature of the bound component and the type of linkage with the protein. In addition we plan to investigate if vinculin inserts also *in situ* into the bilayer, and how such a putative insertion is regulated.

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484 COORDINATED REGULATION IN THE EXPRESSION OF VINCULIN, α ACTININ AND ACTIN DURING THE DIFFERENTIATION OF GRANULOSA CELLS ON ECM AND IN RESPONSE TO GONADOTROPHIC HORMONES

Avri Ben-Ze'ev (1), Abraham Amsterdam (2). (1) Department of Genetics (2) Department of Hormone Research; Weizmann Institute of Science, Rehovot 76100, Israel

We have analyzed the organization and the expression of cytoskeletal proteins involved in determining cell contact and shape in granulosa cells (GC) during their differentiation on an extracellular matrix (ECM), or in response to gonadotrophic hormones. GC grown on ECM acquire an epithelial shape, from multilayered aggregates with numerous gap junctions and display only few actin cables with thin vinculin-containing focal contacts. In contrast, cells grown on plastic form a monolayer of flat cells with few gap junctions, but with numerous stress fibers and large vinculin-positive plaques. Two dimensional gel analysis of ^{35}S-methionine labeled cells revealed a dramatic decrease in the synthesis of vinculin, α actinin and actin in GC on ECM as compared to cells on plastic. GC grown on ECM also produced 26 fold higher levels of progesterone than cells on plastic. Essentially identical changes were obtained in the organization and the expression of vinculin, α actinin and actin by culturing GC on plastic in the presence of gonadotrophic hormones, or compounds such as cholera toxin and forskolin, which elevate cellular cAMP levels. In vitro translation assays with mRNA from control and gonadotropin treated cells revealed a decrease in the translational activity of the mRNAs for vinculin, α actinin and actin in gonadotropin-treated cells, without an effect on the synthesis of the tubulins and of vimentin. RNA blot analysis demonstrated a decrease in both poly(A)$^-$ and poly(A)$^+$ actin mRNA in the cytoplasm of these cells, but the decrease in the poly(A)$^-$ fraction was 5 fold higher than that of the poly(A)$^+$ fraction. The results suggest that the coordinated regulation of the organization and expression of cytoskeletal proteins involved in determining cell contact and shape is part of the programmed alterations which occur during the differentiation of GC, and that the extracellular matrix and cAMP may play an important role in these events.

485 THE MECHANISM OF INTERCELLULAR INTERACTION IN ADHERENS-TYPE JUNCTIONS. B. Geiger and T. Volk Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel.

Adherens-type junctions are specialized cell contacts which are associated at their cytoplasmic faces with actin filament bundles through a vinculin-containing plaque. We have recently identified a 135 Kd surface protein which is specifically associated with cell-cell adherens-type junctions (Volk and Geiger, EMBO J. 10:2249, 1984). This molecule, to which we refer here as adherens-junction specific cell-adhesion molecule (A-CAM), was detected in cardiac myocytes, lens cells, skeletal muscle and brain as well as in a variety of cultured cells. We will provide evidence that A-CAM is involved in the Ca^{2+}-dependent intercellular interaction of adherens junction and that it plays a primary role in the transmembrane induction of cytoskeletal organization. This was concluded from the capacity of monovalent Fab fragments of anti-A-CAM to inhibit junction formation and from the fact that junctions formed in the presence of intact anti-A-CAM become Ca^{2+}-independent.

486 THE MOLECULAR ARCHITECTURE OF ADHAERENS JUNCTIONS

Pamela Cowin, Hans-Peter Kapprell, Werner W. Franke, Division of Membrane Biology and Biochemistry, Institute of Cell and Tumor Biology, German Cancer Research Center, Im Neuenheimer Feld 280, D-6900 Heidelberg, Federal Republic of Germany.

Based on biochemical studies of preparations from bovine snout, desmosomes (maculae adhaerentes) are thought to comprise at least 8 major polypeptides constituents. However, studies based on information obtained with monoclonal antibodies and cDNA clones demonstrate that while some of these components are conserved in all desmosomes, others are restricted, being present only in cells of stratified tissues. Furthermore, certain components which also occur in significant amounts in a soluble form in the cytoplasm are enriched in the plaques of a wider variety of adhaerens junctions, zonulae adhaerentes of epithelial and endothelial cells included. The expression and organisation of these components will be discussed in relation to the cytoskeletal filaments attached.

487 ULTRASTRUCTURE AND IMMUNOELECTRON MICROSCOPY OF FIBROBLAST VENTRAL MEMBRANES: A. Nicol(1), S. Kellie(2) and M.V. Nermut(1). (1) National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K. (2) ICRF Laboratories, St. Bartholomew's Hospital, London EC1A 7BE, U.K.

Ventral membranes of NRK cells prepared by 'lysis-squirting' (see Nermut, 1982) were Pt/C replicated following freeze-drying or critical-point-drying. The identity of substratum adhesions in the replica was determined by correlation with the interference reflection microscopy (IRM) of the same cells before and after lysis-squirting. Large membrane fragments often encompassed many focal adhesions and these were located beneath membrane associated microfilament bundle termini (MFBT). Two types of individual isolated adhesion were found, focal adhesions which in replica comprised the isolated MFBT and its underlying membrane, and clathrin coated membrane patches devoid of any microfilament bundle. These two types had quite different IRM images. Clathrin sheets were often found in lateral association with MFBT and individual isolated focal adhesions. A novel 'linear-coated' cytotic vesicle similar to that described by Peters et al. 1985, was also found. Gold immunolabelling of actin and clathrin gave results in accord with their ultrastructural morphology. The location of vinculin on NRK and chick embryo fibroblast ventral membranes was studied using both monoclonal and polyclonal antibodies. With monoclonal antibody the labelling was concentrated on the MFBT, both on large patches of membrane and on isolated focal adhesions. However, the polyclonal antibody resulted in heaviest labelling on the membrane adjacent to and between the microfilament bundles.

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488 CELL SURFACE EXTENSIONS AND MICROFILAMENT SYSTEM DURING MOUSE EARLY DEVELOPMENT.
Eero Lehtonen and Ilkka Reima. Department of Pathology, University of Helsinki, Haartmaninkatu 3, SF-00290 Helsinki, Finland

The preimplantation mouse lineage, leading to the formation of the trophectoderm and the inner cell mass cells of the blastocyst, apparently depends on cell interactions (c.f. Reima and Lehtonen, Differentiation 30: 68-75, 1985). Cell surface and cytoskeletal structures may be involved in the control of these interactions. We have now studied the possible association between surface morphology and microfilament system in preimplantation embryos, especially during the 8-to-16-cell stage, characterized by the morphogenetically important process of compaction (c.f. Lehtonen, J. Embryol. exp. Morph. 58: 231-249, 1980). In electron microscopy (EM) of early cleavage stages up to the uncompacted 8-cell stage, numerous microvilli were found on all aspects of the blastomeres. In immunofluorescence microscopy with antibodies to vinculin, a cytoskeletal protein associated with sites of microfilament bundle-cell membrane interactions, a submembranous layer of the label was observed in these cells. The vinculin layer included a ring-like concentration in the most peripheral region of each interblastomere contact. During compaction, both microvilli and cortical vinculin showed polar concentration to the outward-facing surface of the blastomeres. Simultaneously, the contacting surfaces of blastomeres showed a reduced number of microvilli, and the quantity of contact-associated vinculin decreased. In late morulae, the cell contacts again showed clear concentrations of vinculin. When compacted embryos were exposed to the microfilament disrupting agent cytochalasin B, they decompacted. Simultaneously, the blastomeres showed a distinct, and strikingly similar, redistribution of microvilli and vinculin-specific fluorescence. These results suggest that a proportion of vinculin may be associated with surface microvilli in these embryo, and our immuno-EM observations support this view. The changes in the distribution of surface extensions and vinculin suggest that these components are involved in the control of preimplantation development.

489 SUBCELLULAR DISTRIBUTION OF VINCULIN IN RESTING AND ACTIVATED PLATELETS.
L. Muszbek, G. Asyee, Róza Ádány, Andrea Horváth. Department of Clinical Chemistry, University School of Medicine, H-4012 Debrecen, POB 40. Hungary

Vinculin is an M_r 130 KD protein which in various cell types is located at the membrane attachment site of microfilaments and has been implicated in membrane-cytoskeleton interaction. We have verified its presence in platelets and isolated platelet vinculin in an electrophoretically homogenous form (Koteliansky et al. FEBS Letts. 1984, 165, 26). Here we show by immunoelectonmicroscopy that in human platelets vinculin is located underneath the membrane and surprisingly also in α granules. It is unevenly distributed in the area of α granules and mainly found in the vicinity of granular membrane. Similar results were obtained in chicken platelets using immunofluorescent staining. These results were confirmed by biochemical studies, as well. By ELISA and immunoblotting techniques the greatest part of vinculin was found in the granular fraction. It is not an integral membrane protein as it cannot be detected in surface or intracellular membrane preparations obtained by free flow electrophoresis. Vinculin is released from the α granules during platelet activation and could be identified in the platelet free supernatant following activation. In resting platelets vinculin is not a cytoskeletal component and it becomes incorporated into the cytoskeleton during thrombin aggregation only after secretion had taken place. On this basis vinculin could be implicated in actin filament-membrane interaction only in the late phase of thrombin activation. The role of α granular vinculin and its release is still to be elucidated.

490 LOCALIZATION OF VINCULIN, TALIN, α-ACTININ AND FIBRONECTIN IN CHICKEN GIZZARD SMOOTH MUSCLE

V.E. Koteliansky, A.M. Belkin, G.N. Gneushev Institute of Experimental Cardiology, USSR Cardiology Research Center, Academy of Medical Sciences, 3rd Cherepkovskaya Str. 15A, 121552, Moscow, USSR

In order to probe the organization of contractile machinery in smooth muscle we have studied the distribution of vinculin, talin, α-actinin and fibronectin, the proteins localized at specialized regions of plasma membrane microfilaments association in different cell types. Antibodies to vinculin, talin, α-actinin and fibronectin were used for localization of these proteins in chicken gizzard smooth muscle by indirect immunofluorescence method. Antibodies to α-actinin decorate mainly intracellular and cell membrane-associated structures of the gizzard smooth muscle cells. These structures seem to correspond to dense bodies and dense plaques. In cross-sections of smooth muscle labeling with antibodies to vinculin and talin reveal a punctate localization at the cell periphery that corresponds to dense plaques. In longitudinal sections labeling with antibodies to vinculin and talin reveal a discontinius periodical structures near cell surface being parallel to long axis of the cell. These structures may correspond to the adhesion plaques previously described in electron microscope studies. Fibronectin has also periodical distribution in chicken gizzard extracellular matrix. It is suggested that in chicken gizzard smooth muscle cells α-actinin is mainly an intracellular F-actin linker in the dense bodies, and that complex of vinculin and talin is involved in the linkage between F-actin and membrane in the dense plaques.

491 IDENTIFICATION OF ACTIN-BINDING PROTEINS IN PLASMA MEMBRANE

Margit Keresztes /1/, Éva Monostori /2/, László Szilágyi /3/
/1/ Institute of Biochemistry, Szeged University of Medical Sciences, H-6701 Szeged, POB. 415, /2/ Institute of Genetics, Biological Research Center of the Hungarian Academy of Sciences, H-6701 Szeged, POB. 521, /3/ Department of Biochemistry, Eötvös Loránd University of Sciences, H-1088 Budapest, Puskin u. 3.

The actin-binding membrane proteins regulate the interaction between the microfilaments and the plasma membrane. Our aim was to detect these proteins in the plasma membrane of liver cells. Plasma membrane was prepared from rat liver. The actin-binding proteins were isolated by affinity chromatography on a column of F-actin bound to CNBr-activated Sepharose 4B. Three polypeptides were identified following SDS-Page in the eluate of the specifically bound fraction. The results of the Western blotting experiments performed directly with the plasma membrane using actin-peroxidase conjugate were in accordance with the ones of chromatography.

492 MEMBRANE-CYTOSKELETON ADAPTIVE CHANGES IN HYPERTONIC MEDIUM.

A.M.FANTIN BOLOGNANI(1),A.M.FUHRMAN CONTI(2),A. FRANCHINI(1),L.BOLOGNANI(1) and B.ZALC(3)
(1)Dep.Animal Biology-Modena(Italy);(2)Dep.Biology and Genetics for Medical Sciences-Milano(Italy); (3)Lab.Neurochimie Hôpital Salpetrière-Paris(France).

An epithelioid cell line(E.U.E.)was tested as suitable to study cytological and biochemical changes during adaptation to salinity.Control cells(C)(grown on 0.137M NaCl)were compared with adapted cells(H) (grown for 10 days on 0.274M NaCl).Both groups were submitted to morphological analyses(SEM,TEM,histochemical reactions)and to specific ultrahistochemical tests for glycoconjugates(Schrével et al.,Progress in Histochemistry and Cytochemistry,14,1-269,1981).Immunocytochemical procedures were also applied using antibodies against cytoskeletal proteins and associated galactolipids.Biochemical tests for membrane and non membrane bound enzymes and for glycolipids were also carried out(Bolognani et al.,Acta histochem.,76, 1-11,1985).The following changes were observed:1)an increase in (H)cell size and an enlargement of cell coat;the plasma membrane appeared more indented;2)microvilli increased in number and size(SEM observations),numerous microfilaments were detectable in their axis by TEM(H cells);3)cytoskeletal intermediate filaments increase in (H) overall around the nucleus(TEM and immunocytochemical tests).An increase is also evident in polar lipids after 25 days of adaptation(sulfatides:90(H) and 20nmoles/10^6cells(C)) and part of them are demonstrable as associated to intermediate filaments(Giuliani et al,In:Contractile proteins in muscle and non muscle cell systems.Biochem.Physiol.Pathol.,E.Alia,M.A.Russo and N.Arena eds., Praeger,Philadelphia,pp.323-333,1985);4)in the intermediate filaments the following components were detected:cheratin,vimentin and gelsolin(immunohistochemical tests);5)microtubules were decreased in (H) cells and generally appeared disassembled as seen by immunocytochemical tests and by TEM.

493 MEMBRANE SKELETON ALTERATIONS AND IgG-RECEPTOR EXPRESSION IN ERYTHROCYTES.

K.-J. Halbhuber, H. W. Meyer, D. Stibenz, W. Linss, H. Oehring. Institute of Anatomy, Friedrich Schiller University, DDR-6900 Jena, Teichgraben 7, German Democratic Republic

Red blood cells (RBC) react very sensitively to environmental influences in vitro and in vivo with structural disturbances of the membrane (membrane alterations). In this connection the expression of IgG receptor sites, which bind autologous IgG is important. After reaching a critical IgG binding threshold RBC were endocytosed by macrophages. The paper checks the hypothesis whether IgG receptor expression is initiated by membrane skeleton alterations (cross linking, loss, dislocation etc.). Model experiments of diamide treated RBC reveal that selective cross linking of spectrin molecules is accompanied by conformational changes of the glycocalyx, redistribution of membrane particles, lipid flip-flop, increased acetylcholine esterase activity and IgG receptor unmasking. It is supposed that a disturbed interaction of the spectrin with band 3 protein during in vivo and in vitro aging of RBC is the crucial mechanism for the IgG receptor expression, which is located at an external domain of the band 3 protein near the glycocalyx.

494 EFFECTS OF CHEMICALLY INDUCED ALTERATIONS OF MEMBRANE-CYTOSKELETON INTERACTION ON THE REACTIVITY OF HUMAN BLOOD PLATELETS

P. Spangenberg (1), Regine Heller (1), St. Gschmeissner (2), U. Till (1) and N. Crawford (2). (1) Institute of Pathological Biochemistry, Medical Academy of Erfurt, 5010 Erfurt, GDR and (2) Royal College of Surgeons of England, Institute of Basic Medical Sciences, WC2A 3PN London, England.

Chemical oxidation of the SH-groups of microfilamental proteins by diamide (azodicarboxylic acid-bis-dimethylamide) is accompanied by alterations of the aggregation behaviour of blood platelets. Incubation of platelets with 0.5 mM diamide for 60 min leads to a complete inhibition of the aggregation ability. In those cells the F-actin is increased, the phospholipid content of isolated cytoskeletons is decreased and the structural organization of the cytoskeleton is changed to electron dense zones in the centre of the cell in contrast to the lose networks of filaments in unactivated platelets. Incubation of platelets with 0.1 mM diamide leads via deaggregation (5 min) and inhibition of aggregation (30 min) to a normalization of the aggregation behaviour after 60-120 min of incubation with the SH-reagent due to reduction of certain disulfide-linked polymers of microfilamental proteins by the cellular repair mechanism. In parallel the cellular F-actin returns to a normal value as well as the phospholipid content of the cytoskeleton. Only the structural feature of the cytoskeleton does not return to normal probably due to the still existing change in the F-actin-pools (cytoskeletal and cytoplasmic) within the cell. Structural organization of cytoskeletons in the centre of the cell after diamide treatment and the decrease of phospholipids in the cytoskeleton are considered to be signs of an altered membrane-cytoskeleton interaction.

495 ORGANIZATION AND EXPRESSION OF GENES ENCODING INTERMEDIATE FILAMENT (IF) PROTEIN.

Hans Bloemendal, Guillaume van Eijs, Richard v.d. Heuvel, Frank Pieper, Frans Ramaekers and Wim Quax. Department of Biochemistry, University of Nijmegen, The Netherlands.

The five major classes of IFs are expressed in a highly specific way. Cytokeratins are typical for epithelial cells, vimentin for mesenchymal tissue and most cells in culture, desmin for myogenic differentiation, neurofilaments for neurons and glial fibrillary acidic protein (GFAP) for glial cells. The exact rules that govern this specialization are hitherto unknown. In order to study this cell-specific expression in some more detail we cloned the vimentin and desmin genes with the aim in mind to eventually transfer them into cells in which they are normally not expressed.

Sequence analyses revealed a very close relationship in structural aspects of the two IF genes. For instance both genes have 9 exons and 8 introns at corresponding sites. Surprisingly, the isolated desmin gene is upon transfection faithfully expressed in non-muscle cells. This means that at least under the conditions of our experiment there is no expression barrier due to the heterologous cell type used (e.g. expression of the desmin gene in transformed hamster lens cells which normally synthesize only vimentin as sole IF protein subunit).

We have also constructed hybrid genes comprising the promoter of vimentin followed by a number of 5' vimentin exons that were linked to 3' desmin exons and, alternatively, the desmin promoter with 5' desmin exons coupled to 3' vimentin exons.

After transfection both "artificial" genes were expressed in appropriate culture cells. Apparently the hybrid proteins (named vimine and desmintin by us) were synthesized and incorporated into filamentous structures undistinguishable from the *bona fide* IF cytoskeleton.

496 THE EVOLUTION OF ACTIN AND ACTIN GENES

J. VANDEKERCKHOVE & K. WEBER
Laboratory of Genetics, State University of Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium
Max-Planck Institute for Biophysical Chemistry, D-3400 Göttingen, F.R.G.

Actin is an extremely conserved protein. It is expressed in different variants (isoforms). Warm-blooded vertebrates, express six different isoactins. They could be classified into two striated muscle actins (a skeletal muscle and cardiac form), two smooth muscle actins (a vascular and visceral form) and two non-muscle or cytoplasmic actins. No amino acid differences were observed when actins from the same tissue were compared in different mammalian or avian species.

In a study on the evolutionary origin of the different mammalian isoactins, we have analysed isoactin expression in various chordate and invertebrate tissues. This study was carried out using a previously described actin typing procedure which is based on a two-dimensional paper electrophoretic analysis of the ^{14}C-carboxymethylated NH_2-terminal tryptic peptide used as a marker peptide. In addition, electrophoretic analysis of the partial acid hydrolysis fragments of the marker peptide, allowed to predict the order of the acidic residues located at the very NH_2-terminal region of the polypeptide. Data from these analyses were further confirmed by total amino-acid sequence analysis of actins isolated from "key-species" in actin evolution.

Invertebrates (five different phyla were studied), express only actins which are homologous to the non-muscle vertebrate isoforms, rather than to their vertebrate counterparts (this also includes invertebrate muscle actins).

The actin type which may be considered as the vertebrate muscle actin precursor, appears with the origin of chordates. In early chordates (Agnathes) it is the only muscle actin (expressed both in heart and epaxial muscle and probably also in smooth muscle cells). It is very different form the invertebrate proteins but differs only in three residues from human cardiac actin. Later in evolution, (with Condrichthyes) this ancestral form duplicated again to give rise to a specific striated muscle and smooth muscle form. In reptiles both forms duplicated again to give rise to the known four warm-blooded vertebrate muscle actins.

A similar duplication event occured in the evolution of the non-muscle actins. Originally there was one cytoplasmic actin type, strikingly different from the ancestral invertebrate type, and very similar to the mammalian beta-actin type. This form duplicated in reptiles with the appearance of the mammalian non-muscle types.

Amphibia pose a special problem in this relatively simple evolutionary process. Thus, Xenopus laevis expresses simultaneously two striated muscle actins (both expressed in heart and skeletal muscle), one smooth muscle actin and not less than three different non-muscle actins. This situation becomes even more perturbed when further analysis in different amphibia showed an unexpected species specific variation of the non-muscle actins.

The evolutionary tree of the isoactins, based on protein-chemical data will be discussed in terms of the known structures of invertebrate and vertebrate actin genes and also in correlation with the known post-translational processing of the different actins.

497 ORGANIZATION OF CYTOKERATINS AND THEIR GENES: PRINCIPLES OF DIFFERENTIAL EXPRESSION OF A MULTIGENE FAMILY.

Werner W. Franke[1], José L. Jorcano[1,2], Thomas Magin[1], Bernhard Bader[1], Jürgen Franz[1], Valentino Romano[1], Manfred Blessing[2], Anita Knapp[1], Mechthild Hatzfeld[1], Thomas Achtstätter[2]. [1]Division of Membrane Biology and Biochemistry, Institute of Cell and Tumor Biology, German Cancer Research Center, Im Neuenheimer Feld 280, D-6900 Heidelberg, and [2]Center for Molecular Biology, University of Heidelberg, Im Neuenheimer Feld 282, D-6900 Heidelberg, Federal Republic of Germany.

The cytokeratins are a family of polypeptides of intermediate-sized filaments (IF) which are specifically expressed in epithelial cells (and certain precursor cells) and their malignantly growing derivatives. They share certain immunological epitopes, amino acid sequence homologies and conformational principles and can be divided into two subfamilies, the acidic (type I) and basic (type II) polypeptides which share less than 30 % sequence homology with each other. Type I and type II polypeptides form coiled-coil heterotypic tetramer complexes containing two chains of either subfamily, which then assemble into protofilaments and IF. Analyses of cDNA and genomic clones have shown that the various cytokeratin genes also display several identical exon-intron patterns (some of the intron positions are also shared with other IF proteins) as well as differences in the exon-intron organization, the latter being mostly located in the gene region corresponding to the non-α-helical "tail" portion of the protein. Moreover, similarities and differences in introns and upstream sequences will be discussed in relation to possible principles of the regulation of the specific expression of different combinations of type I and type II polypeptides in different epithelial cells.

498 REGULATION OF THE TUBULIN MULTIGENE FAMILY DURING BRAIN DEVELOPMENT.

Irith Ginzburg and Uriel Z. Littauer, Department of Neurobiology, Weizmann Institute of Science, Rehovot, Israel.

The diversity of microtubule functions and the temporal employment of microtubules through cell division, growth and differentiation suggests that the expression of tubulin genes may be tightly regulated. Microtubules are particularly important in the brain where they are involved in cell differentiation, migration and synaptic transmission.' In mammalian DNA, hybridization experiments with labeled tubulin cDNA probes have revealed the presence of α- and β-tubulin multigene families. However, the number of functional genes is unknown, and indeed several rat α-tubulin pseudogenes were identified and sequenced. To study the expression of an individual member of the tubulin gene family, specific cDNA probes were constructed. Two α-cDNA clones were isolated and their nucleotide sequence examined. These two clones share high homology within the coding region; however, the 3'-untranslated coding regions are highly divergent. A strong interspecies homology exists in the 3'-untranslated region when compared with specific α-tubulin isotypes sequences from other mammals, i.e. hamster and human cells. The interspecies homology suggests functional requirement for that region. Both α-tubulin genes encode for an identical size mRNA of 1.8-k.b.

The nucleotide sequence of a rat brain β-tubulin has also been determined. The coding region shows a high homology when compared to chicken and human β-tubulin sequences. However, the 3'-terminal coding end shows a high degree of divergence and no homology is observed at the 3'-untranslated region. Comparison of the derived amino acid sequences from different species demonstrates that the amino acid changes are not randomly distributed, but rather there are several conserved and two highly variable regions common to β-tubulin polypeptides from various sources. Three β-tubulin mRNA species are present in rat brain: A dominant neuronal 1.8-k.b. species and two minor species of 2.6 and 2.9-k.b. respectively. By using two synthetic oligonucleotide probes complementary to the carboxy-terminal divergent region and the amino-terminal conserved region, it was demonstrated that the three mRNAs are distinct species, which are developmentally regulated. The level of the 1.8-k.b. mRNA species increases till the age of 12 days thereafter its level decreases. The 2.9-k.b. mRNA is an early neuronal mRNA species, while the 2.6-k.b. mRNA is a late neuronal species which is detected at 30 days of rat brain development. The data illustrate that there is a differential expression of the β-tubulin multigene family during rat brain development which may suggest different functions for the various β-tubulin isotypes.

We have developed a specific binding assay for the interaction of ^{125}I-labeled MAPs with cleavage peptides derived from tubulin. It was shown that MAP2 and TAU factors will interact with peptides located near the carboxyl terminus of β-tubulin. It also appears that this region has diverged and evolved more rapidly throughout evolution than the other constant regions of tubulin. It is possible that the binding domain close to the 3'-end of tubulin and the corresponding site on MAPs underwent molecular coevolution and thus provide a mechanism to generate new control mechanisms for microtubule assembly.

The specific probes and synthetic oligonucleotides were used for *in situ* hybridization studies of rat cerebellum which show differential tubulin mRNA levels in identified cell types.

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499 HUMAN VIMENTIN GENE : EXPRESSION IN LYMPHOCYTES AND BURKITT'S LYMPHOMA CELLS.

A. Lilienbaum, V. Legagneux, J. Perreau, M.M. Portier, K. Dellagi and D. Paulin. Unité de Génétique cellulaire, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France.

In the lymphoid series of cells, B lymphocytes show a well developed vimentin network. B lymphocytes obtained from patients suffering from Burkitt lymphoma (fresh cells as well as established cell lines) do not exhibit such a network (1, 2). *In vitro* translation of poly A$^+$ RNA in a rabbit reticulocyte cell free system has shown that vimentin is a major product of normal lymphocytes but is absent from Burkitt's lymphoma lymphoblastoid cells. A human vimentin genomic clone had been obtained and hybridized to total and poly A$^+$ RNA from human fibroblasts, lymphocytes and Burkitt's lymphoma cells. One band corresponding to 2000 bases was revealed with RNA from fibroblasts and lymphocytes but not with RNA from the Burkitt's lymphoma cell lines DAUDI, RAJI, BJAB, JBL2, J1. The absence of RNA would suggest an alteration at the transcriptional level or a rapid degradation of the vimentin mRNA. The Burkitt's lymphoma cell lines show a characteristic translocation involving the distal q24 of the chromosome 8 and either the chromosome 14, 2 or 22. Similar results were obtained with lymphoid cells derived from patients suffering from the Langer-Giedion syndrome with a deletion on the chromosome 8. The fact that chromosome 8 is deleted or translocated in these two cases argues in the favour of a regulatory mechanism involving vimentin.

(1) K. Dellagi et al. (1983) The EMBO Journal, 2, 1509-1514.
(2) K. Dellagi et al. (1984) J.Nat.Cancer Inst., 73, n° 1, 95-100.

500 CHANGES IN THE ORGANIZATION OF NON-EPITHELIAL INTERMEDIATE FILAMENTS AS INDUCED BY TRIETHYL LEAD CHLORIDE. Hans-Peter Zimmermann (1), Ulrich Plagens (2), Costas E. Vorgias (2), Peter Traub (2), (1) Institute of Cell and Tumor Biology, German Cancer Research Center, D-6900 Heidelberg, and (2) Max-Planck-Institute for Cell Biology, D-6802 Ladenburg/Heidelberg, Federal Republic of Germany

The in vivo effect of triethyl lead chloride (TriEL) (10^{-6}M to 10^{-8}M) on the organization of non-epithelial intermediate filaments (vimentin-, desmin-, and neurofilaments) was studied by indirect immunofluorescence microscopy employing different mammalian cell lines. The in vitro effect of TriEL on filament formation as well as on the structure of preformed filaments was investigated by electron microscopy. TriEL induces perinuclear coil formation of intermediate filaments in SV40-transformed human fibroblasts, baby hamster kidney (BHK-21) cells, and neuroblastoma (Neuro-2a) cells. The rearrangements observed are not correlated with significant changes of the microtubular system as tested by double labeling of both filament systems. The effect of TriEL is reversible. Assembly of intermediate filaments in vitro is disturbed in the presence of TriEL such that only short filaments and different kinds of fragments are formed. On the other hand, if preformed filaments are incubated in the presence of TriEL, unraveling of fibers into subfilament strands is observed. The results indicate both an alteration of the integrity of non-epithelial intermediate filaments by TriEL as well as a reduction of their capacity to reconstitute normally.

501 STRUCTURAL HOMOLOGY AND SITE-SPECIFIC PHOSPHORYLATION OF INTERMEDIATE FILAMENT ASSOCIATED PLECTIN IN VARIOUS CULTURED CELL LINES. Harald Herrmann and Gerhard Wiche, Institute of Biochemistry, University of Vienna, A-1090 Vienna, Austria.

Plectin, a protein of M_r 300,000, was originally isolated from rat glioma C6 cells together with the 1% TritonX-100/0.6M KCl insoluble intermediate filament fraction. In cytoskeletal preparations plectin is phosphorylated by associated kinases at molecular sites identical to those radiolabeled in vivo by $^{32}P_i$ (Herrmann & Wiche, JBC, 258, 14610, 1983). We now examined the structure of C_6 plectin in comparison to that of 300 kDa proteins present in intermediate filament preparations from various other cell lines (CHO, BHK-21, 3T3, HeLa, A-431) by one-dimensional peptide mapping. V8 protease digests of in vitro labeled proteins (reductive methylation) yielded very similar patterns with only minor differences. This confirmed the immunological relationship between the various plectins as revealed by antisera raised against C_6 plectin. By two-dimensional polyacrylamide gel electrophoresis and chromatofocusing followed by peptide mapping, plectin was shown to consist of a single polypeptide chain focusing between pH 5.7 and 4.0 depending on its state of aggregation. Peptide mapping of plectin from various cell lines that had been labeled in vivo with ^{32}Pi demonstrated that the phosphorylation sites were generally located at the end(s) of the polypeptide chain. Thus, all the plectins examined apparently have conserved primary structure and, furthermore, are phosphorylated in molecular domains potentially important for the binding to other proteins. Together with additional electron microscopy and gel overlay data to be presented, the data suggest that plectin interconnects intermediate filaments and is engaged in the binding of intermediate filaments to cellular membranes.

502 CYTOSKELETAL PROTEINS OF TRYPANOSOMA BRUCEI AND THE ORGANIZATION OF THEIR GENES Richard Braun, Thomas Seebeck, Martin Imboden, Valentin Küng and André Schneider. Institute of General Microbiology, University of Bern, Baltzer-Strasse 4, 3012 Bern, Switzerland

Trypanosoma brucei is a protozoon and causes sleeping sickness in humans as well as the nagana disease in cattle. Of all cytoskeletal proteins tubulin is by far the most prominent and is located in both subpellicular and flagellar microtubules. Some tubulin is also detected free in the cytoplasm. Both alpha- and beta tubulin have been characterized and they are similar to tubulins of vertebrates. In vivo the C-terminal tyrosine is removed and added on again. The rate of this modification varies for different cellular compartments. Two dimensional gel electrophoresis has revaeled a further modification. The genes for tubulin are arranged in a single large cluster, in which alpha and beta genes alternate regularly. There are about 15 repeats of each, located on one of the larger chromosomes. The mRNAs for both alpha and beta tubulin have a 35 nucleotide miniexon at their 5'end. The sequences of the miniexons are identical to those found on the mRNAs coding for variable surface glcoproteins. A further cytoskeletal protein has been characterized and designated P60. This protein may be involved in attaching the subpellicular microtubules to the cell membrane. It is expected to obtain further information on the P60 protein through the cloning and sequencing of its gene.

503 CHARACTERIZATION OF THE RECEPTOR FOR INTERLEUKIN 1 IN THE HUMAN LEUKAEMIC CELL LINE K 562.
Klaus Resch (1), Michael Martin (1) and David H. Lovett (2). (1) Abt. Molekularpharmakologie, Medizinische Hochschule Hannover, D-3000 Hannover 61, FRG, and (2) Div. Nephrology, Washington University, Seattle, USA.

Interleukin 1 (IL-1) was induced in murine macrophage tumors (P388D1) and purified to apparent homogeneity. The cytokine concentration dependently inhibited the proliferation of mouse T cell lymphomas, as well as the human leukaemic cell line K 562. The cytostatic action of IL-1 was not associated with cytotoxicity and appeared to be irreversible. FACS analysis of IL-1 treated K 562 cells showed a rapid decrease of transferrin receptors and, a more delayed, increase in HLA-A,B,C antigen density. Neither PGE nor interferon appeared to mediate these effects, suggesting that it was caused immediately by the receptor for IL-1, most likely involving the induction of a differentiation step, associated with the loss of autonomous growth.

To characterize the receptor for IL-1, highly purified plasma membranes of K 562 were isolated. At 0^{o} C IL-1 induced the phosphorylation of a 41 kDa membrane protein in a time and concentration dependend manner. The resistance to treatment with alkali, as well as the analysis of phosphoamino acids revealed that IL-1 specifically induced phosphorylation on tyrosine residues. The protein was also weakly phosphorylated without IL-1 being present; this phosphorylation did not involve tyrosine. At 37^{o} C the protein was rapidly dephosphorylated, which could be inhibited by vanadate and zincate. Affinity labelling with the ATP-analogue fluorosulfonylbenzoyladenosine showed that the 41 kDa protein possessed the ATP binding and cleaving site. Crosslinking experiments with disuccinimidyl suberate and [^{125}I]-IL-1, or with IL-1 and plasma membranes, which were phosphorylated in response to the monokine, identified the 41 kDa protein as the receptor molecule.

We conclude from this that the IL-1 receptor in the human leukaemic cell line K 562 is a transmembrane protein of 41 kDa, which possesses a tyrosine specific protein kinase with an autophosphorylating capacity. In a recently isolated subclone of K 562, which was resistant to growth inhibition, IL-1 also failed to induce phosphorylation of the IL-1 receptor, suggesting, that this molecule mediates the biological functions of IL-1.

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504 EARLY RESPONSE PATTERN ANALYSIS OF THE MITOGENIC PATHWAY IN LYMPHOCYTES
T.R.Hesketh, A.N.Corps, J.C.Metcalfe, J.P.Moore, J.D.H.Morris, G.A.Smith
Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

In a systematic attempt to define the mitogenic pathways from G_o to S phase in mouse thymocytes, we have analysed the early biochemical responses to mitogens. Although the primary response to each mitogen can be distinguished by the pattern of secondary responses it initiates, there is substantial overlap in these responses. Our aim is therefore to determine whether there is early convergence on a pathway common to different mitogens that is defined by a sequence of responses obligatory for progression from G_o to S phase. The 'dual-signal' hypothesis for the mitogenic stimulation of thymocytes is a simple version of a common mitogenic pathway. It proposes that the T-cell receptor activates the pathway via the breakdown of phosphtidylinositol (4,5)-bisphosphate, generating a Ca signal (from the release of inositol (1,4,5)-trisphosphate) and activating protein kinase C (by the release of diacylglycerol). This hypothesis is based on the comitogenic action of the Ca ionophore A 23187 and the phorbol ester 12-o-tetradecanoyl phorbol 13-acetate, which is assumed specifically to activate protein kinase C. However, detailed analysis of the coupling between some of the early responses, including the Ca and pH signals, phosphatidyl (4,5) -bisphosphate metabolism, protein kinase activity, c-fos and c-myc gene activation and general metabolic stimulation, indicates clearly that the hypothesis is inadequate to account for the initiation of the normal mitogenic pathway in thymocytes.

505 TUBULIN, CALMODULIN, AND VIMENTIN REORGANIZATIONS DURING THE MITOGENIC STIMULATION OF MOUSE SPLENIC LYMPHOCYTES. David L. Brown, Roberto Campos, and Micheline Paulin-Levasseur. Department of Biology, University of Ottawa, Ottawa, Canada K1N 6N5.

We have used double immunofluorescence staining with polyclonal and monoclonal antibodies to tubulin, calmodulin, and vimentin in various combinations to examine the changes in distributions of their respective antigens in mouse splenic lymphocytes stimulated by concanavalin A. During stimulation there are changes in the organization and microtubule assembly activity of the centrosome and up to a 5-fold increase in the numbers of assembled microtubules (Schweitzer and Brown, Biol. Cell 52, 147, 1984). In unstimulated lymphocytes vimentin is detected as a filamentous array which parallels the sparse microtubule system extending from the centrosome. Calmodulin, in unstimulated cells, is diffusely distributed in the cytoplasm. During stimulation calmodulin becomes associated with the centrosome and some of the microtubules coincident with increase in microtubules assembled from the centrosome. At this stage vimentin is reorganized to a single fluorescent spot, with no obvious filamentous array, localized at or near the centrosome. As the stimulated cells enter mitosis vimentin again reorganizes into filamentous arrays focussing on the mitotic centrosomes, and subsequently forms a cage around the spindle. The calmodulin-centrosome interaction established during stimulation remains throughout mitosis, as has been described for other animal cells. These observations, and others of lymphocytes treated to disassemble microtubules (colcemid), and alter centrosome structure (low temperature), or promote assembly of microtubules from the centrosome (taxol), indicate that a cold-labile calmodulin-centrosome interaction occurs during lymphocyte stimulation. This interaction is temporally correlated with, and may regulate, the changes in centrosome structure and the reorganizations of the vimentin filament and microtubule components of the lymphocyte cytoskeleton during stimulation and mitosis.
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506 STEPS OF LYMPHOCYTE ACTIVATION DEPENDENT OF IONIC TRANSPORT.
Jean-Claude Mani, Jacques Dornand, Jean Favero. Laboratoire de Biochimie des Membranes, ER CNRS 228. ENSCM, 8 rue de l'Ecole Normale, F-34075 Montpellier, France.

The mitogenic effect of lectins on T cells involves three main steps: 1) helper T cells in resting G_0 state, upon binding of the lectin, go to a G_1 activated state; when these activated cells are stimulated by interleukin-1 (IL-1) secreted by macrophages, they synthesize and release interleukin-2 (IL-2); 2) at the same time, in a separate activation pathway, some T cells, either helper or suppressor/cytotoxic, stimulated by the lectin and IL-1, express on their surface receptors for IL-2; 3) the binding of IL-2 to its receptors induces DNA synthesis and cell proliferation. Because mitogens or antigens can stimulate the expression of IL-2 receptors without concomitant secretion of IL-2, different transmembrane signals or intracellular messengers may threrefore be involved in these pathways. Numerous messenger systems have been implicated in the response to mitogenic lectins; we report here our studies on the role of ionic transport. Most of the events leading to lymphocyte stimulation seem controlled by ion transport mechanisms, including ion channels, carriers and active transport devices such as the Na-K-ATPase.

1. Role of Calcium uptake. Intracellular free calcium concentration is very low (about 100 nM), while total intracellular calcium is about 0.4 mM and extracellular calcium generally about 1 mM. This intracellular free calcium comes either from extracellular calcium (calcium channels which are blocked by drugs such as verapamil, nifedipine or the phencyclidine analog GK_3) or from the release from intracellular calcium stores, under the effect of inositol triphosphate. The Ca-ATPase which hydrolyzes extracellular ATP is not involved in an active efflux of calcium against its concentration gradient; however this enzyme appears involved in lymphocyte activation: its specific inhibition by several ATP analogs prevents Con A-induced thymocyte stimulation. We found that drugs blocking the calcium channel inhibit mouse or human lymphocyte stimulation; some inhibitors were found more efficient for human cells than for mouse cells (verapamil, GK_3), while nifedipine is more inhibitory for mouse splenocytes. The inhibition of lymphocyte stimulation is complete if GK_3 is added during the first 6 hrs of the culture; if added after 24 hrs, no more inhibition occurs. The production and release of IL-2 in the supernatant of cells stimulated by Con A is measured after 24 hr culture by its ability to support the growth of an IL-2-dependent T cell line. We found that the inhibition of IL-2 synthesis by GK_3 parallels its inhibition of DNA synthesis. It appears that calcium uptake is involved in the synthesis and release of IL-2 by stimulated T cells, like it is involved in the mechanism of many secretory cells. On the contrary calcium is neither involved in the expression of IL-2 receptors, nor in IL-2-induced lymphocyte proliferation.

2. Role of potassium uptake. Twenty years ago Quastel and Kaplan have shown that the blastogenic transformation of human lymphocytes by PHA was inhibited by low concentrations of ouabain through the inhibition of the transport Na-K-ATPase. We found that harmaline, another Na-K-ATPase inhibitor, inhibits both PHA and Con A-stimulated human lymphocytes. At concentrations as low as 0.1 μM ouabain inhibits the proliferation of human lymphocytes regardless of how the cells are stimulated (antigens, lectins, oxydative mitogens, mixed lymphocyte reaction), and at any stage of the stimulation. Actually this drug, after binding to the enzyme, was shown to inhibit several parameters of the proliferative response, but surprisingly we found that it does not inhibit IL-2 production. On the contrary we found that IL-2 production is higher in human cells cultured in the presence of ouabain, even at concentrations which completely inhibit lymphocyte proliferation (0.1-0.2 μM); the maximal increase (3-6 times) is reached with 0.05 μM ouabain, a dose leading to partial mitogenic inhibition. We found that ouabain does not stimulate IL-2 production by Jurkat cells which do not need IL-1 to be stimulated, which might mean that IL-2 production is not directly affected by ouabain and is independent of potassium uptake. Indeed we have shown that the increase of IL-2 production in ouabain-treated human lymphocytes arises from an increased synthesis of IL-1 by mononuclear cells upon ouabain treatment.

Ouabain is able to induce IL-1 synthesis by mononuclear cells even in the absence of mitogenic lectin; this effect is not prevented or reversed by high potassium concentrations and appears unrelated with ouabain-induced Na-K-ATPase inhibition. The inhibition of lymphocyte proliferation by ouabain is not related to inhibition of IL-2 production but to the blockade of the expression of IL-2 receptors and to the inhibition of IL-2 induced cell proliferation; both effect are directly linked to the inhibition of potassium uptake. 0.05 μM ouabain inhibits (65%) the binding of exogeneous IL-2 to Con A-stimulated human lymphocytes; 0.1 μM ouabain leads to complete inhibition. On the other hand the proliferation of a human IL-2-dependent T cell line induced by lectin-free IL-2 is completely blocked by 0.1 μM ouabain.

3. Role of sodium transport. The sodium-proton exchange channel was recently detected in lymphocytes from blood and thymus. In other cell types similar antiports are activated by growth factors, suggesting a role in mitogenesis. We found that amiloride, a specific inhibitor of this sodium channel is a potent inhibitor of lymphocyte stimulation induced by PHA, with half maximal inhibition for 60 μM amiloride, and complete inhibition for 130 μM; this inhibition is reversible by extensive washing of the cells. Amiloride effects parallel those of ouabain: stimulation of IL-2 production, inhibition of the expression of IL-2 receptors. We are presently running experiments to see if amiloride, like ouabain, prevents also the stimulation by IL-2 of human IL-2dependent cell lines.

The data reported here evidence the important role of ionic transport in lymphocyte stimulation by mitogens: calcium is necessary for the synthesis of IL-2 but not for the expression of IL-2 receptors or for IL-2 induced proliferation; the blockade of sodium and potassium transport does not prevent IL-2 synthesis but prevents the expression of IL-2 receptors and (at least for potassium) IL-2-induced proliferation. However the relationships between the intracellular concentrations of these ions make difficult the interpretation of the role of each individual ion in the mechanism of lymphocyte proliferation.

507 ADP-RIBOSYLATION AND REGULATION OF CHROMATIN FUNCTION

H. Altmann , Institut für Biologie, Forschungszentrum Seibersdorf, A-2444 Seibersdorf, Österreich

Molecular biological data suggest,that ADP-ribosylation regulate the activation, but also inactivation of cellular processes including DNA replication,DNA repair, transcription of genes differentiation and transformation.

Eucaryotic chromatin structure varies as a function of DNA sequence and gene activity dependent also on binding of poly(ADP-ribose) to histon and non-histone proteins.

The chromatin bound poly(ADP-ribose)-polymerase is inactive,unless it is bound to DNA. Interspersed repeated DNA sequences,scattered along eukaryotic chromosomes might function as origins of DNA replication including DNA amplification and activate the poly(ADP-ribose)-polymerase more efficiently than total bulk DNA. Experiments using modulators of poly(ADP-ribose)-synthesis in C 57 bl mice showed a pronounced effect on activation of lymphocyte subsets. Dependent on the treatment schedule, poly (ADP-ribose)-polymerase inhibitors showed an enhancement or an inhibition of transformation processes.

508 FUNCTIONAL DOMAINS OF THE T LYMPHOCYTE PLASMA MEMBRANE: MOLECULAR TARGET OF PHYSIOLOGICAL AND PHARMACOLOGICAL REGULATORS OF LYMPHOCYTE ACTIVATION

M.Szamel,P.Berger,M.Goppelt,M.Kövecses,M.Martin L.Szollár,K.Resch and J.Somogyi Institute of Biochemistry,I.and of Pathophysiology, Semmelweis Univ. Med.Sch.,Budapest,Hungary and Institute of Molecular Pharmacology, Medical School,Hannover,FRG

Resting lymphocytes are activated by antigens and mitogens to differentiate and simultaneously to grow and divide. How in molecular terms the ligand-receptor interaction triggers a cellular response still remains to be elucidated. Upon the binding of antigens or mitogens to cell surface receptors the plasma membrane itself undergoes numerous changes. As a possible explanation for this pleiotypic membrane response there is the assumption that a receptor-linked amplification is operative in the plasma membrane, underlying the many functional changes observed. In resting T lymphocytes the plasma membrane contains functional domains formed by tight association of the triggering mitogen receptor with some enzymes, (Na+K)ATPase,lysolecithin acyltransferase,etc.,embedded in a phospholipid milieu different from the bulk membrane.As an immediate consequence of the mitogen-receptor interaction the receptor-linked enzymes are activated.Due to the activation of lysolecithin acyltransferase the saturated fatty acids of plasma membrane phospholipids are replaced by polyunsaturated ones.As the newly formed phospholipid molecules with higher degree of unsaturation are rapidly dispersed the entire membrane matrix is altered.The increase in the content of polyunsaturated fatty acids in the membrane phospholipids modulates other membrane functions.One of such effectors - or several in concerted action- then creates the intracellular messenger(s) responsible for cell activation.By linking different membrane functions to the triggering receptor and thus amplifying a signal within the plasma membrane we propose that plasma membrane phospholipids form an integral part in the stimulus-response coupling in the activation of lymphocytes.Several physiological and pharmacological inhibitors of lymphocyte activation,like serum lipoproteins,apoproteins,ouabain (and analoga),as well as cyclosporin A interfere with the early activation of receptor-coupled lysolecithin acyltransferase and thus with the elevated turnover of plasma membrane phospholipid metabolism,suggesting

that functional domains of the plasma membrane are involved in the plasma membrane dependent control of cell growth and division.

509 Calcium permeability of the T lymphocyte plasma membrane: counteraction of phorbol ester and A23187

Péter Csermely, Márta Szamel and János Somogyi
Inst. of Biochemistry I., Semmelweiss University Medical School, Budapest

The intracellular calcium concentration (Ca_i) of T lymphocytes was measured using the fluorescent indicator quin2. Different ionophores effectively enhanced the Ca permeability of the plasma membrane. The effective concentration of the ionophores required for permeabilization increased in the order of ionomycin, A23187 and X537-A (lasalocid-A). 12-O-tetradecanoyl-phorbol-13-acetate (TPA) in submicromolar concentrations did not change Ca_i.

The addition of TPA immediately before the A23187-permeabilization did not alter the Ca ionophoretic effect of A23187. However prolonged incubation with TPA decreased the efficiency of A23187 permeabilizing the plasma membrane for calcium ions. This effect was concentration and time dependent being maximal at TPA concentrations higher than 10 nM with a preincubation time of 1.5 hours.

TPA induced relative A23187 insensitivity is most probably not due to a direct effect of TPA on the ionophore as it is concentration and time dependent. Moreover the fluorescence and fluorescence polarisation of A23187 as well as the energy transfer between the tryptophane groups of the membrane proteins and A23187 showed no significant change during incubation with TPA. These results indicate that membrane fluidity changes or A23187 immobilization also do not play a prominent role in the explanation of the phenomenon. However the supposed intracellular heavy metal content of T lymphocyte might be a possible source of the TPA induced relative insensitisation towards A23187.

510 LONIDAMINE EFFECT ON CULTURED GERM CELLS

M. Galdieri, Istituto di Istologia ed Embriologia generale, Univ. 'La Sapienza', Rome, Italy

Lonidamine (Lon), a dichlorinated derivative of indazole-3-carboxylic acid has been used as antispermatogenic substance (De Martino et al,Exp Mol Pathol,23, 1975,1; Chemotherapy,27,1981,27). Its effect on the Sertoli cells secretory activity (Lobl et al,Chemotherapy,27,1981,61) and on the oxygen consumption of the germ cells (Floridi et al,Exp Mol Pathol,35,1981,314) is well known. More recently an antiblastic effect of Lon has been postulated and an effect on the DNA-dependent DNA polymerase on tumor cells has been evidentiated (Concolino et al, 2° Int Conf. on Progress in Cancer Research,San Remo,Italy,1985). We have studied Lon effect on the duplication of germinal and somatic cells of the seminiferous epithelium. Cultures of seminiferous epithelium explants were prepared from prepubertal Wistar rats (Palombi et al,J Reprod Fert, 57,1979,325). In this 'in vitro model' germ cells remain attached to the Sertoli cells and are able to incorporate ^{3}H-Thymidine. Lon (5 ug/ml, gift of F.Angelini Research Institute) was added to the culture medium and 30 min after Lon administration ^{3}H-Thymidine (2 uCi/ml) was added. After labelling the cells were washed, collected and the amount of the radioactivity incorporated into TCA precipitable material was evaluated. The results obtained indicate that Lon cause a 50-60% reduction of thymidine incorporation into the germ cells. Autoradiography of control and treated cells showed a reduction of the autoradiographic grains per nucleus after Lon administration. Peritubular cells treated with the same dose of Lon incorpotated the same amount of thymidine as control cells. Our data indicate that Lon directly affects germ cells metabolism.

511 INVOLVEMENT OF CYTOSKELETON IN EARLY STAGES OF AXOLOTL OOCYTE MATURATION.

Jean GAUTIER and Jean-Claude BEETSCHEN. Laboratoire de Biologie générale, Unité Associée CNRS n° 675 Univeristé Paul Sabatier, 118 route de Narbonne 31062 TOULOUSE CEDEX FRANCE.

Axolotl (*Ambystoma mexicanum*) oocyte provides a very useful model-system to study the early stages of maturation until germinal vesicle breakdown (GVBD). During *in vitro* maturation, GVBD occurs around the tenth hour after progesterone addition (at 18°C). Germinal vesicle first migrates towards the animal pole, this germinal vesicle migration (GVM) becomes noticeable from five hours after hormone addition and reaches its maximum at height hours.

The aim of this study was to know what generates GVM during progesterone-induced maturation and what elements of the cytoskeleton could be involved for GVBD. We performed *in vitro* maturation of inverted oocytes and with cytoskeleton antagonists. So we were able to obtain early GVM and GVM in abnormal position. We must point out two important findings : first is that GVM is an active phenomenon as revealed by inverted oocyte experiments; second is that GVM is regulated by microtubules as shown by antagonists experiments. An immunocytochemical analysis of cytoskeleton modifications during progesterone-induced maturation is now under investigation.

512 CALCIUM AND POLYPHOSPHOINOSITIDES: THEIR DISTRIBUTION ALONG THE MEMBRANES OF THE SPERM HEADS.

Giovanna Berruti, Emilia Franchi. Dipartimento di Biologia, Università di Milano, 20133 Milano, Italy.

Ejaculated boar spermatozoa, previously incubated in a rigorously Ca^{++}-free medium, were exposed to Ca^{++} for different incubation times and processed for the detection of Ca^{++} localization by a pyroantimonate technique. The distribution of polyphosphoinositides, anionic phospholipids natural constituents of membrane known to bind Ca^{++}, was investigated using a specific cytochemical probe, i.e., neomycin conjugated with horseradish peroxidase. The "in situ" localizations thus obtained revealed: 1) the occurrence of a Ca^{++}-induced release of calcium from the nonmitochondrial intracellular store, i.e., the outer acrosomal membrane; 2) this fusigenic membrane was the elective site of the neomycin/peroxidase labeling. In addition, the cytochemical staining elicited a striking change in the morphology of the membrane suggesting the possibility of the occurrence of a lipid phase-transition. When assayed for the presence of a phospholipase C-like activity, the detergent extract obtained from boar spermatozoa exhibited substantial amount of p-nitrophenyl-phosphorylcholine hydrolyzing activity. The results, on the whole, allow us to put forward the hypothesis of a relationship between Ca^{++} and polyphosphoinositides turnover in the events triggering the acrosome reaction, the exocytotic process peculiar to mammalian spermatozoa.

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513 "EFFECT OF RABBIT ANTISERUM TO CERTAIN PLASMA MEMBRANE ASSOCIATED PROTEINS OF BOAR SPERM ON IN VITRO FERTILIZATION". Maria Ivanova, Iglika Batova and Margarita Mollova. Institute of Biology and Immunology of Reproduction and Development of Organisms, Bulgarian Academy of Sciences, Sofia 1113,Bulgaria

A fraction of soluble weakly bound plasma membrane proteins is isolated from hypotonically treated boar sperm and shown by SDS-PAGE to be represented by several polypeptides of molecular weight ranging 13-20 kD, 45-60 kD and 67-80 kD. The titre of antibodies to these proteins, detected in ELISA, is 1: 51200. The effect of the IgG fraction of the antisera on sperm/egg interaction is tested in vitro fertilization system. Following incubation of spermatozoa, undergoing in vitro capacitation, with antibodies to membrane proteins, the motility and ability for penetration in homologous and heterologous ovocytes, is affected. Incubation of porcine ovocytes in different IgG concentrations exert no influence on sperm binding, but inhibits the penetration through zona pellucida. Inhibition of the penetration is also observed in zona-free hamster ovocytes. The role of certain membrane associated proteins in sperm/egg recognition and binding is discussed.

514 IMMUNOCYTOCHEMICAL LOCALIZATION OF CALMODULIN IN INTACT AND ACROSOME-REACTED BOAR SPERM. Marina Camatini, Giuliana Anelli, Antonella Casale, Angela Piotti Dipartimento di Biologia, Universita' di Milano, Italy

Localization of calmodulin on intact and acrosome-reacted ionophore A23187 induced boar sperm has been performed with indirect immunofluorescence (IIF) and immunoelectronmicroscopy (IEM). IIF: the semen was processed in a calcium free buffer; sperm suspension, smeared on slides, fixed in absolute methanol at -20°C was reacted with sheep or rabbit anticalmodulin and subsequently with FITC rabbit antisheep IgG, or FITC goat anti-rabbit IgG. IEM: samples were fixed in 2% paraformaldehyde and 0,2% glutaraldehyde; dehydration, Lowicryl K4M embedding and polymerization under ultraviolet were carried out at -35°C. Sections, mounted on nickel grids, were labelled with rabbit anticalmodulin with pA-G15. The specificity of the antibodies to calmodulin was evaluated by immunoblotting; the antibodies reacted with sperm calmodulin and specifically precipitated only a single band in the 17 kDalton region. The results obtained with IIF are in agreement with previous reports. IEM was performed both with the en bloc staining technique and the protein A-colloidal gold immunostaining on Lowicryl K4M sections. Calmodulin has been evidenced in the acrosomal content and in the equatorial and postacrosomal regions of intact sperm. Calmodulin appears associated with the plasma and the outer acrosomal membranes in acrosome-reacted sperm and it is maintained in the equatorial and postacrosomal regions. These results provide further evidence that, also in mammalian sperm, multiple classes of calmodulin-binding proteins may be present.

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515 ULTRASTRUCTURAL CHANGES IN THE OLIVE POLLEN GRAIN (OLEA EUROPAEA L.) DURING ITS DEVELOPMENT. Fernández Fernández,M.C., M.I.Rodríguez García. Estación Experimental del Zaidín. CSIC. Granada, Spain.

In higher plants male gametogenesis takes places in the pollen grain formed by the division of a haploid microspore which gives rise to a vegetative and a generative cell. The pollen grain begins to mature at this point, a process which concludes with the dehiscence of the anther and release of the mature pollen grain. Studies on the ultrastructural aspects of olive pollen grain development are scarce (1,2,3) hence the purpose of the present study was to analyze ultrastructural changes taking place from the early microscope period until the splitting of the anther and reflecting metabolic and functional transformation during this period. The following stages were selected as development reference points in olive pollen (Marteño variety): young microspore inmediately after release from the tetrad; vacuolated microspore; newly-formed bicellular grain (generative cell near pollen cell wall); pollen with generative cell housed entirely within the vegetative cell cytoplasm (semimature grain); and mature grain just before release from the anther. Pollen grain volume increases throughout development although most spectaculary during the microspore vacuolization. Vacuoles begin to appear in the cytoplasm in the free microspore, reaching maximum volume before postmeiotic mitosis takes places. Once the bicellular grain has formed, the large central vacuole begins to break up into smaller and more numerous fragments, eventually leading to the complete disappearance of the vacuole. The plastids increase in number throughout development while their starch content reaches its maximum in the bicellular grain, decreasing thereafter to disappear completely in the mature grain. The lipid bodies on the other hand grow steadily up until the mature stage, as does the rough endoplasmic reticulum replete with proteinaceous material. These ultrastructural changes indicate a high degree of metabolic activity. The mature olive pollen grain is filled with storage reserves, like a seed at maturity.

(1) Pacini, E. & M. Cresti (1977), Planta 137, 1-4. (2) Pacini, E. & B.E. Juniper (1979), New Phytol. 83, 157-163. (3) Pacini, E. & G. Casadoro (1981), Protoplasma 106, 289-296.

516 SENSITIVITY OF LEYDIG, SERTOLI AND GERM CELLS TO GONADAL STEROIDS. Vladimir Pantić, Dušan Gledić. Department of Histology and Embryology, Faculty of Veterinary Medicine, 11ooo Beograd, Bulevar JNA 18, Yugoslavia.

Leydig and Sertoli cells, as gonadal steroids producing cells, the rate of germ cells multiplication and spermatogenesis in rat testes as reaction to gonadal steroids administered at different stages of development have been investigated and the obtained results compared with the sensitivity of corresponding cells to estradiol+progesterone injected into the piglets on the first day of life. The testes pieces isolated from both examined species, fixed with 4% glutaraldehyde, postfixed with 1% osmic acid, and embedded in araldite, were examined as semithin and ultrathin sections. The results obtained using light and electron microscopy might be briefly summarized as follows: as a result of regressive changes in both Leydig and Sertoli cells of rats, clearly expressed soon after estradiol treatment, the steroids and other products of these cells showed a tendency of permanently decreased activities pronounced on spermatogonia mitosis and changed the ratio of the basal and adluminal region, altering meiosis and spermatogenesis. The prolonged characteristics of the effects of these steroids via brain peptides, PRL and gonadotropic hormones provoked sterility in rats which was more expressed if they were treated during the neonatal than later periods of development. On the contrary, the testes of piglets neonatally treated with estradiol+progesterone showed signs of retarded spermatogenesis clearly pronounced at the onset of the first meiotic division.

517 EXAMINATION OF HORMON SELECTIVENESS OF EMBRIONAL LEYDIG CELLS IN RAT
O. Dobozy /1/, O. Brindak /2/, V. Zagurovszkij /2/, G. Csaba /1/.
/1/ Semmelweis University of Medicine, H-1445. Budapest, P.O.B. 370, and
/2/ Institute of Endocrinology and Hormone Chemistry Research, Kharkow, Artemstr. 10, USSR

The hormone receptors develop in an early period of ontogeny through a process of maturation, during which their binding capacity and specificity increase. Following our earlier investigations pregnant rats were treated from the 11th to 15th or from the 16th to the 20th day of gestation with chorionic gonadotropin, TSH or prolactin. 24 hour after the last treatment the animals were exterminated and the testes of the embryos were embedded for routine histology.On the hematoxilin-eosin stained sections it was determined the volume of the nuclei of the Leydig cells. To test the synthetic activity of the Leydig cells after acridine--orange staining with microspectrofluorimetry it was measured the luminescence of the Leydig cells. From the blood of the embryos it was determined the testosteron concentration of the sera, too.

Our results show that both of the two glycoprotein hormones,chorionic gonadotropin and TSH, enhanced significantly the activity of Leydig cells, while the polypeptide type prolactin had only a moderating effect. These results agree well with our hypothesis, that hormones of similar molecular structure may bind to the same binding sites of the embryonal rat Leydig cells, in which the maturation of gonadotropin-receptors not yet finished.

518 CORRELATION OF GERM CELLS AND SERTOLI CELLS IN ONTOGENESIS OF WISTAR RATS
O.I.Brindak, B.V.Aleshin, O.V. Volkova, V.S.Sukhorukov, S.B. Tarabrin, O.Dobozi.
Kharkov Institute of Endocrinology and Hormone Chemistry, USSR, Semmelweis University of Medicine, Budapest, Hungary.

To study the dependence of the Sertoli cell differentiation on the presence of germ cells, the postnatal development of testis after its sterilization in the intrauterine period by twofold injection of myelosan (bisulfan) to pregnant females was investigated. The normal embryogenesis of testis during the whole prenatal ontogenesis was also studied. It was found, that on the I6-I7th day of intrauterine embryo development a high synthetic RNA activity is observed in the gonocyte plasma of testis disclosed by the method of microspectral luminescent analysis, and also rise of alkaline and acid-phosphatase activity, the gonocyte cytoplasma accumulates much glycogen and other mucopolysaccharides. All these processes in the primary germ cells precede enhancement of the Sertoli cell differentiation. Beginning from the I8th day of prenatal ontpgenesis the synthetic activity in the gonocytes decreases, part of them undergoes dystrophy and decay. In experiments with bisulfan administration it is shown, that beginning from the second month of postnatal life sone ultrastructural changes are observed in the Sertoli cells. Such changes are absent in the control and separate areas of spermatic canals of experimental animals with preserved spermatogenesis. The problem is considered of mutual effect of germ cells and Sertoli cells in the ontogenesis of rats.

519 FINE-STRUCTURAL AUTORADIOGRAPHIC LOCALIZATION OF ADENOSINE-^{3}H INCORPORATION DURING FERTILIZATION IN MAMMALIAN EGG. V. Kopečný/1/, A. Pavlok/2/, M. Tománek/3/. /1/ Research Institute of Animal Production, CS-251 61 Praha-10-Uhříněves, /2/ Institute of Animal Physiology, Czechoslovak Academy of Sciences, Líběchov, and /3/ Research Institute of Animal Production, Nitra, Czechoslovakia.

Adenosine-^{3}H is taken up 1 000 times faster than uridine-^{3}H by one cell mouse embryo allowing the detection of RNA synthesis and cytoplasmic polyadenylation at this early stage of development/CLEGG and PIKO, Nature 295,348,1982/. Presented communication extends these observations by attributing adenosine-^{3}H incorporation to defined subcellular compartments of the in-vitro fertilized, zona-free hamster egg.

In nuclear structures, the earliest labelling was seen in early male pronuclei /homologous and heterologous/ in a time interval before the expected DNA synthesis and was interpreted as the earliest detected transcription, starting probably shortly after chromatin decondensation.

In ooplasm, statistically significant labelling was attributed to mitochondria and to cytoplasmic bilaminar lamellae, specific for eggs of some rodents. Other localizations may not be excluded. Ooplasmal labelling was interpreted, at least partly, as the sites of polyadenylation of stored maternal RNA the localization of which may be revealed in this way.

520 FERTILIZATION-INDUCED ENDOCYTOSIS IN XENOPUS EGGS. Giovanni Bernardini, Roberto Stipani and Marco Ferraguti. Dipartimento di Biologia, Università di Milano, 26 Via Celoria, I-20133 Milano, Italy.

Following activation, Xenopus egg plasma membrane undergoes a radical reorganization; to continuosly monitor it, membrane capacity (C_m) has been measured during activation (2). C_m increases in the first minutes following activation, then gradually decreases; the increase is due to the addition of cortical granule membrane to the plasma membrane (2), the decrease corresponds to a reduction of surface area accomplished by an endocytotic mechanism (1).

To visualize fertilization-induced endocytosis, eggs were fertilized in medium containing 0.1% Lucifer yellow. Zygotes, fixed at different times after fertilization, contain fluorescent vesicles in the cortex. The fluorescent vesicles are asymmetrically distributed. Endocytosis occurs mostly at the animal pole, although some vesicles are present also in the vegetal hemisphere. The vesicles are present along the cleavage furrow of the first division and are not disappeared yet 170 min after insemination, at 16 cell stage. During the early development of the embryo, the endocytotic vesicles do not migrate towards preferential positions or move deeper in the cytoplasm, but remain localized in the areas of the cortex where endocytosis occurred; in fact, even at 16 cells stage they are frequent in the animal and rare in the vegetal pole of the embryo.

(Supported by the C.N.R. sub-project "Contraception")

(1) Bernardini G., Ferraguti M. and Peres A. (1986) Gam. Res. (in press).
(2) Peres A. and Bernardini G. (1985) Pfluger Arch. 404: 266-272.

521 TRANSCRIPTIONAL ACTIVITY AND PATTERNS OF mRNA PREVALENCE DURING EARLY MOUSE EMBRYOGENESIS. L. Pikó and K. D. Taylor. Developmental Biology Laboratory, Veterans Administration Medical Center, Sepulveda, California 91343, U.S.A.

Several lines of evidence indicate that protein synthesis in the one-cell fertilized mouse egg is carried out mostly if not entirely by maternally inherited components. However, during the two-cell stage (the second day of development) a new pattern of protein synthesis emerges which is dependent on RNA synthesis by the embryonic genome. Studies in our laboratory have shown that the mouse egg is very rich in poly(A)+ RNA but that the bulk of this RNA is degraded in the two-cell embryo. There is a very low level of stable RNA synthesis in the one-cell zygote but all the major classes of RNA are synthesized from the two-cell stage onwards, resulting in a six-fold increase in total RNA content and the number of poly(A)+ mRNA between the two-cell and blastocyst stages. We have recently explored the changes occurring during this period in the prevalence of some individual mRNAs, using a cDNA clone library derived from two-cell embryos. Recombinant plasmid DNA from 76 clones selected at random was dotted onto nitrocellulose filters and hybridized with ^{32}P-labeled cDNA probes produced from eggs and embryos at the two-cell, eight-cell and blastocyst stages. A positive reaction was obtained in about one-third of the clones, estimated to represent moderately abundant (>0.1% of the mRNA population) to highly abundant sequences. About one-half of these sequences had a similar prevalence at all stages examined, but the prevalence of the other half increased considerably from the two-cell stage onwards, suggesting that their expression is regulated during cleavage. These observations provide direct evidence that a new pattern of genetic activity is initiated at the two-cell stage of development.

522 CLEAVAGE AND EMBRYOGENESIS IN **XENOPUS** ARE INHIBITED BY WEAK BASES.

Dennis J. Webb and Michel Charbonneau. Laboratoire de Cytologie Expérimentale, Université de Rennes I, Rennes, France.

Evidence has been rapidly accumulating in recent years that indicates a major role for intracellular pH and Ca^{2+} in the regulation of many cell processes particularly at the time of cell activation. A change in the intracellular activity of either one of these ions is invariably associated by a change in the other. In this study we have found that the weak bases NH_4^+ and procaine inhibited first cleavage of **Xenopus** eggs at an extracellular pH close to their respective pKa's. Cleavage was disrupted to varying degrees depending on the concentration of the weak base employed, the duration of exposure and the time at which eggs were exposed with respect to the onset of cleavage. Although 100% of eggs fertilized in 5 mM NH_4^+ only about 35% subsequently divided. However, at higher concentrations even fertilization was inhibited. Disruption of cleavage was most noticeably associated with furrow regression. The furrow often began to form in treated eggs at the equatorial edges rather than at the animal pole region. A nonpigmented strip eventually appeared in this region. At the time of second cleavage this gave the embryo a "hot cross bun appearance". Milder treatment permitted early cleavage but seemed to disrupt the usual synchronous divisions while at submillimolar concentrations marked exogastrulation was induced. The early development of sea urchins and starfish were affected in an apparently similar manner by the same weak bases. The similar effect on early development of different weak bases at high pH suggest the involvement of a rise in intracellular pH in the mode of action.

523 ALPHA ACTININ DISTRIBUTION AND EXTRACELLULAR MATRIX PRODUCTS DURING SOMITOGENESIS AND NEURULATION IN THE CHICK EMBRYO.

James W. Lash, David S. Ostrovsky, Balraj Mittal, and Joseph W. Sanger. Department of Anatomy, University of Pennsylvania, Philadelphia, PA (J.W.L., B.M., J.W.S.) and Department of Biology, Millersville University, Millersville, PA (D.S.O.).

A discrete stage in two different morphogenetic processes has been examined employing fluorescently labelled alpha-actinin as a probe to localize native alpha-actinin and antibodies to localize fibronectin and collagen type I. The stage of somitogenesis examined is the transition from the compact mesenchymal somitic mass to the epithelial somitic vesicle (i.e., epithelialization of the somite). The stage of neurulation examined is the transition from the relatively flat neuroepithelium to the approximation of the neural folds. Before these morphogenetic movements begin, the neuroepithelium is sitting upon a basal lamina and interstitial collagen and the somite is surrounded by a meshwork of interstitial collagen. During both of these processes, the cells become narrowed at their apices in the region of the tissue that is becoming concave and alpha-actinin is localized in the apices. The localization of intracellular alpha-actinin and extracellular fibronectin and the distribution of collagen suggest that there is a coordinated appearance and distribution of these molecules that is temporally associated with these discrete morphogenetic events.

524 STRUCTURE OF OTOLITH ORGANS OF XENOPUS LAEVIS TADPOLES DEVELOPING IN WEIGHTLESSNESS.

Dmitrii Lychakov, Sechenov Institute of Evolutionary Physiology and Biochemistry, Academy of Sciences USSR, 194223, Leningrad, Thorez pr., 44, USSR.

Xenopus laevis tadpoles (stages 45 and 46) developed for 8 and 9 days in special biocontainers at 15°C in the orbiting space station "Salut-6" had three maculae (utricular, saccular, lagenar). Based on morphology of ciliary tufts, four hair cell types were identified, similar to types A, B, C, E in the bullfrog tadpoles (Li, Lewis, 1979). In X.laevis tadpoles type D cells were absent while in the bullfrog tadpoles this type proved to be predominant type in the saccular maculae. No continuous layer of immature type A hair cells could be found at the periphery of the maculae. The character of polarization of cells in the utriculus and sacculus of X.laevis tadpoles was the same as in the bullfrog. The lagena of X.laevis tadpoles had more complicated pattern of polarization than that in the bullfrog. Substantial qualitative changes in the maculae and in the otolith membrane have not been observed. The morphometric analysis indicated an increase of size of the utricular otoliths by 1.3 times as a result of exposure to weightlessness ($26.3 \pm 5.3 \times 10^4$ μm^3, n=22, and $34.2 \pm 7.3 \times 10^4$ μm^3, n=14, p=0.99). There was a tendency for a greater asymmetry between the left and right otoliths in the same tadpole. The saccular and lagenar maculae were covered by a single otolith. There are no substantial differences between saccular and lagenar otoliths.

525 *INVOLVEMENT OF Na^+ PUMP ACTIVITY DURING RETINOIC ACID-INDUCED DIFFERENTIATION IN THE HUMAN PROMYELOCYTIC CELL LINE HL-60. B. Geny, A. Ladoux, I. Krawice and J.P. Abita. INSERM 204 Hôpital Saint Louis 2, Place du Dr Fournier 75475 PARIS CEDEX 10 FRANCE*

The HL-60 cell line, established from a patient suffering from a promyelocytic leukemia was shown to be able to differentiate towards granulocyte-like cells in the presence of retinoic acid (RA). In 5 days RA at 10^{-6}M induces the differentiation of more than 80 % of cells (NBT+).

After addition of the drug, early and late changes in the sodium pumps (number of molecules and/or activity) were observed. During the first 12 hours following addition of RA, no modification in the number of ouabain binding sites - i.e. in the number of pump molecules-occurred ; however the turn-over of the enzymes is greatly enhanced, reaching 180 % of the initial value after 8 hours. Later, and paralleling the appearance of morphological and functional differentiation, we observed a decrease in the number of ouabain binding sites per cell, (not accompanied by a modification in the affinity for the drug) as well as an increase in the K^+ transport per Na^+ pump molecule.

Ouabain, the specific inhibitor for the Na^+ pump, has no effect on the HL-60 cell differentiation or is toxic when used continuously at concentrations greater than 5×10^{-8}M. At 3×10^{-8}M for 8 hours, ouabain added with 2×10^{-7}M RA, induces an early stimulation of the pump reaching a maximum after 2 hours, whereas RA alone gives a peak in the pump activity after 8 hours. Moreover, in these conditions, ouabain potentializes the effect of RA on differentiation, inducing 50 % of NBT+ cells after 3 days while RA alone (at 2×10^{-7}M) leads to 30 % NBT+ cells in 4-5 days.

In this work, we demonstrate that : 1) addition of RA to HL-60 cells provokes i) an early stimulation of the Na^+ pump which peaks after 8 hours ; ii) later a decrease in the number of the Na^+ pump molecules which reflects differentiation itself ; 2) there exists a time course relationship between the Na^+ pump stimulation and differentiation.

526 NEUROTRANSMITTER RECEPTORS AND STIMULUS-SECRETION COUPLING IN A HUMAN NEUROBLASTOMA CELL LINE DURING DIFFERENTIATION. C. Gotti(1), E. Sher(1), M.P. Abbracchio(2), M. Bozzi(1), D. Cabrini(1), D. Fornasari(1) and F. Clementi(1). (1) CNR Center of Cytopharmacology, Department of Pharmacology, University of Milano, and (2) Institute of Pharmacology and Pharmacognosy, University of Milano, Milano, Italy.

Neuroblastoma cell lines have many advantages for the investigation of specific neuronal properties and especially for studying the mechanism of differentiation. We performed our studies on the IMR32, a human neuroblastoma cell line grown in vitro. Control cells have a spindle shape with few short neurites. They contain the catecholamine-synthesizing enzyme, tyrosine-hydroxylase (TH), high levels of the neurotransmitters Adrenaline, Noradrenaline, Dopamine, and particularly high levels of Serotonin. However, they have very few dense-core vesicles in the cell body and in neurites. While these cells do not express and adrenergic and dopaminergic receptors, they have high affinity muscarinic binding sites, as measured by ^{3}H-QNB and ^{3}H-N-Methylscopolamine. They also express ^{125}I- Bungarotoxin binding sites with pharmacological characteristics of a nicotinic receptor. Adenosine analogs stimulate cAMP production (10 folds) with potencies characteristic of the A2 adenosine receptor. The treatment of IMR32 cells with both 5-Bromodeoxyuridine and dibutyryl-cAMP induces morphological differentiation, although with a different pattern. In each case there is an increase in the content of dense-core vesicles, of TH activity and of neurotransmitters. Differentiation with 5-Bromodeoxyuridine increases both muscarinic and nicotinic receptors two-fold without changes in Kd, while tratment with dibutyryl-cAMP induces an increase of only nicotinic receptors. With both treatments, on the other hand, adenosine receptors are down-regulated during differentiation. Nor control nor differentiated cells release exogenous uptaken or endogenous neurotransmitters, after treatment with a battery of secretagogues as Carbachol, K^+, Ca^{++} ionophores, and -Latrotoxin.

For all these reasons IMR32 cells can be considered a suitable model for studying neurotransmitter receptor regulation and stimulus-secretion coupling in human neurons during differentiation.

527 INDUCTION OF MORPHOLOGIC DIFFERENTIATION IN A HUMAN MELANOMA CELL LINE BY 2,5 HEXANEDIONE.

W. Malorni, G. Formisano and A. Molinari. Department of Ultrastructures, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy.

The effects of 2,5 Hexanedione (2,5 HD) on a cultured human melanoma cell line (JR8) were explored. The addition of the toxical at noncytolitic concentrations (0.08% and 0.16%) for 24 and 48 hours resulted in an irreversible inhibition of cell proliferation. Cessation of melanoma cell proliferation was accompanied by wide changes of morphological features of cells still adhering to the substrate. Incubation with the toxical at 0.16% concentration seemed to induce a differentiative process mainly characterized by a noticeable increase of cell protrusions. Melanoma cells, losing their bipolar appearance, increased cell size and developed long dendritic and axon-like processes sometimes ramified in distal portions. Electron microscopic observation allowed to establish that a change in perinuclear polarization of organelles occurred after 2,5 HD treatment. In fact a regular arrangement of mitochondria, melanosomes and cytoskeletal elements was detectable in dendritic and axon-like protrusions. Furthermore, immunocytochemical studies confirmed an involvement of microtubules and actin network within cell prolongations. After the differentiative process a necrotizing effect occurred inducing a progressive loss of viable, dendritic cells after 4 or 5 days. Incubation with cyclic AMP after 2,5 HD treatment seemed to increase the survival rate of neuronal-like cells, while no significant changes were detectable in control cells. On the basis of our results JR8 melanoma cells seem to be a highly suitable model for the study of some neurotoxins (i.e. 2,5 HD related, and newly synthesized compounds) and their growth inhibiting and differentiating capability.

528 DIFFERENTIATION OF A HUMAN MYELOMONOCYTIC CELL LINE

A.B. LYONS Dept. Microbiology and Immunology, The University of Adelaide, GPO Box 498 ADELAIDE, SOUTH AUSTRALIA, 5000

The human myelomonocytic cell line, RC2A (Bradley, 1983) was cultured from the peripheral blood of a patient with chronic myelomonocytic leukaemia. The predominant cell type are early monocytes, as determined by morphological and functional criteria. Recently we have shown that conditioned medium from phytohaemagglutinin stimulated human peripheral blood mononuclear cells PHA-LCM has the effect of inducing RC2A cells to differentiation is accompanied by a loss of proliferative potential, surface marker changes, morphological alteration and appearence of histochemical markers associated with macrophages.

Murine antiserum raised against uninduced RC2A cells, after absorbtion to an autologous B cell line, has also been shown to exert differentiative as well as proliferative effects on RC2A and on macrophage precursors in the bone marrow progenitor cell (CFU-C) assay. From these investigations, we have developed a strategy involving bioassay techniques to screen monoclonal antibodies produced in this laboratory for the ability to mimic or block the effects of biological molecules active in these systems.

529 ISOLATION AND PRELIMINARY CHARACTERIZATION OF A SPONTANEOUSLY DIFFERENTIATING TERATOCARCINOMA CELL LINE

L. Székely. Institute of Biology, University Medical School, H-4012 Debrecen, Hungary

Spontaneously differentiating murine teratocarcinoma cells were isolated from a tumor which was obtained by inoculation of nullipotent F_9 cells into the abdominal cavity of a baby mouse. This new cell line (F_9SD) seems to be very useable in experiments studying the relationship between differentiation and the loss of malignancy. These cells are able to produce differentiated derivatives *in vitro* as well as *in vivo*. The stem cell feature was checked by alkaline phosphatase positivity detection. The first sign of differentiation is the disappearance of enzyme activity which is followed by morphological changes. First of all endoderm, trophectoderm and neuroectoderm cells appear containing altered intermediate filaments and displaying characteristic morphological features. The differentiating capacity can be modified with the alteration of plating number, the inhibition of attachment to surface of Petri dish, and biologically active compounds. Anchorage-independently growing cells form aggregates (embryoid bodies). These aggregates are able to modify the morphology of human laryngeal carcinoma (HEp2) cells growing in monolayer. Upon direct cell-cell contact between the embryoid bodies and HEp2 monolayer, from the cells of the latter, long, axon-like processes are developed, and dividing activity is lost. Among other circumstances, using medium preincubated with F_9SD cells or mixing suspensions of HEp2 + F_9SD single cells in any proportion, the mentioned alteration of the epitheloid cells was not observed.

Our results may be indicative of an eventual importance of spatial situation of the cells playing role in the induction of cell differentiation *in vitro*.

530 CHANGES IN THE GLYCOSYLATION PATTERN DURING EMBRYONIC DEVELOPMENT OF MOUSE KIDNEY.

L. Laitinen, I. Virtanen and L. Saxén. Department of Pathology, University of Helsinki, Haartmanink. 3, SF-00290 Helsinki 29, Finland.

Distribution of lectin-binding sites during mouse kidney development in vivo and in vitro was studied by using fluorochrome- and peroxidase-coupled lectins.

The uninduced mesenchymal cells of the early metanephros reacted widely with lectins recognizing N-linked saccharides. Upon induction, PNA-positivity and to a lesser extent, SBA-positivity rapidly appeared in the induced mesenchymal cell aggregates.

The podocytes of the early glomerules expressed heterogenously terminal galactosyl and N-acetyl galactosaminyl moieties. These sites disappeared later and were apparently covered by sialylation as judged by the concomitant emergence of a strong WGA-positivity and by the appearance of these sites upon neuraminidase treatment. During in vitro development such a covering sialylation took place only partially. Also endothelial cells showed a transient expression of terminal galactosyl moieties which became cryptic upon maturation. In adult mouse kidney, proximal and distal tubules presented distinct lectin-binding patterns. Terminal fucosyl-residues, characteristic of mature proximal tubules, appeared during day 13 of development. Similarly, DBA-positivity emerged in the maturing collecting ducts by day 13. On the other hand, α-galactosyl moieties, visualized with GSA-I-B_4-isolectin, were characteristically present only in endothelia in adult mouse kidney cortex but in embryonic kidney they were also found in collecting ducts and podocytes.

The present results show that the compartmentalization of glycoconjugates, as seen in adult mouse kidney, is acquired in a sequential manner during development. Both in glomerules and endothelial cells some of the terminal saccharides appear to be transitionally expressed during development. Such a sequential appearance of the mature type of glycosylation pattern probably reflects functional maturation of the nephron.

531 GLYCOPROTEIN BIOSYNTHESIS IN ISOLATED HEPATOCYTES DURING FETAL DEVELOPMENT

S.Leoni, L.Conti Devirgiliis, S.Spagnuolo, M.T.Mangiantini-Department of Cellular and Developmental Biology-University of Rome "La Sapienza"-Piazzale A.Moro - 00185 ROME - ITALY

The glycosilation of proteins may be considered as an integral part of the process of cell differentiation and of the development and morphogenesis of different tissues. For this purpose we have studied using rat hepatocytes isolated at different stages of development (18-20, 21-22 days of intrauterine life, and the first day after the birth), the incorporation of ^{14}C glucosamine and ^{3}H galactose in the cellular glycoproteins and its control by glucagon and dibutyril cyclic adenosine monophosphate (db cAMP). The percentage of ^{14}C glucosamine assumed by the cells which is successively incorporated in clorophorm/methanol/H_2O extract and in tricloroacetic acid insoluble fraction appears to be in the adult hepatocytes about 25% while in the perinatal period is only 10%. This probably indicates that the transfer of glucosamine on the dolichol linked oligosaccharide and subsequentely on the proteins is impaired during fetal development. Similarly the ^{3}H galactose incorporation is clearly lower in the fetal and neonatal period, when the incorporation of ^{3}H leucine is greatly enhanced. This fetal characteristic is present also in the regenerating liver and in the hepatoma which are interesting models of proliferating tissues. In the experimental conditions used by us there is no evident modification of glucosamine and galactose incorporation by dbcAMP and glucagon, suggesting that such substances have not a short term effect on this metabolic pattern.

532 CYTOKERATIN AND VIMENTIN INTERMEDIATE FILAMENTS AS MARKERS FOR EARLY RAT SERTOLI AND GRANULOSA CELLS

Kim Fröjdman (1), Jorma Paranko (2), Ismo Virtanen (3), Lauri J. Pelliniemi (1). (1) Laboratory of Electron Microscopy, (2) Department of Anatomy, University of Turku, SF-20520 Turku, (3) Department of Pathology, University of Helsinki, SF-00290 Helsinki, Finland

Developing gonadal ridges of 11 to 15-day-old male and female rat fetuses were cryo-cut for the immunocytochemical analysis of cytokeratin and vimentin intermediate filaments. Reference plastic sections were made for light and electron microscopy. At the age of 11 days, no proper gonadal ridge was yet detected and the prospective area was negative for cytokeratin. The reaction for vimentin was strong in the mesenchyme but weak in the coelomic mesothelium. The presence of discontinuous mesothelial basement membrane (BM) was confirmed by laminin (provided by Dr. J-M. Foidart) immunocytochemistry. At the age of 12 days, thickenings of the mesothelium invaded the mesenchyme of the gonadal ridge. First signs of sexual differentiation were observed at the age of 12 days. Simultaneously with the formation of the BM around the organizing testicular cords, basal accumulations of cytokeratin appeared in vimentin-containing Sertoli cells. Similarly, female granulosa cells started to express cytokeratin at the same age, but throughout the study, their organization was distinctly different from that of Sertoli cells. The results suggest that the expression of cytokeratin in the basal cytoplasm of Sertoli and granulosa cells is related to the formation of the BM and the separation of the epithelial gonadal cords from the interstitium. Coexpression of vimentin and keratin in the fetal Sertoli and granulosa cells provides a useful marker for the identification of these cells in various experimental and pathological conditions.

533 EVIDENCE FOR THE REGULATION OF ENDOCRINE FUNCTION IN HUMAN FETUS BY ONSET OF SPECIFIC MORPHOFUNCTIONAL DIFFERENTIATION OF THE ENDOCRINE ORGANS.

B. Bukulya (1), V.I. Altuchova (2). (1) Institute of Experimental Medicine, Hungarian Academy of Sciences, H-1450 Budapest, POB 67, Hungary, and (2) Institute of Human Morphology, Academy of Medical Sciences USSR, Moscow, Curupy 3.

To understand endocrine regulation at early stages of the differentiation of endocrine organs, it is very important to know the specific intracellular signs of endocrine function. The aim of our study was to localize enzyme activity in the human fetal hypothalamus which takes part in the transformation and elimination of neurohormones. We have attampted also to demonstrate enzyme activity in the peripherial endocrine organs showing their capabilities to respond to specific stimuli. Non-specific esterase (NSE) activity was demonstrated by Davis's(1) method. Adenylcyclase (AC) activity in hypophysis, adrenal cortex, thyroid glands and gonads was demonstrated by Howel's(2) method. In the region of the paraventricular and arcuate nuclei in the human fetal hypothalamus, NSE activity was observed in granules of different diameter and with low electron dense center within the karyoplasm of some neural cells. The enzyme activity was represented by high electron dense fine grains. In the other regions of the hypothalamus compact granules of NSE activity with high electron density were localized in the growing neural processes and in the karyoplasm of the differentiating neural cells. AC activity was localized mostly on the cell membrane of the fetal hypophysis and peripherial endocrine organs as early as 7-8 weeks. Our data suggest the possibility that the human hypothalamo-hypophyseo-adrenal axis has some degree of specific function at 7-8 weeks of gestation. 1.,Davis,R. et al., J.Cell Biol., 34:157(1967), 2.,Howel,S.L. et al., J.Histochem.Cytochem., 20:873(1972).

534 MUTANT CELLS OF THE RAT PITUITARY GLAND.
M.Japundžić, V.Lačković, S.Milićević, V.Bumbaširević. Institute of Histology, Medical Faculty, Višegradska 26, 11000 Belgrade, Yugoslavia.

In Previous investigations we have established that the mutation can affect the cytodifferentiation of the pituitary pars intermedia cells leading to the appearance of a number of operationally useful phenotypic markers allowing a new insight into the real nature of the cells (Experientia 41, 570, 1985). In the present study we investigated the effects of gene mutation on the cytologic and cytochemical characteristics of the anterior pituitary cells. We observed that the cytologic properties and histologic patterning of the endocrine subpopulation of the pituitary cells remained unaltered. However, the mutation did enhanced phenotypic expression of genome at the level of folliculo-stellate and marginal types of cells resulting in the production of the typical melanosome structure.

It was concluded that the aberant melanocytic differentiation of the folliculo-stellate and marginal cells reflects neuroectodermal determination of their embriological precursors and that both cell types derived from the cephalic part of the neural crest.

535 CHANGES OF FREE HISTONES IN CHICK TESTICULAR AND OVARIAN CELLS AFTER EMBRYONIC AND/OR NEONATAL TREATMENT WITH DIETHYLSTILBESTROL /DES/ OR ALLYLOESTRENOL /AE/.
P.T. Sótonyi, O. Dobozy, G. Csaba, Semmelweis University of Medicine, H-1445. Budapest, P.O.B. 370. Hungary

On the ninth day of incubation AE and DES were injected into the yolksac of chicken embryos. At hatching some of the animals were also treated. After 24h animals were been exterminated and the gonads fixed for histology. The phosphormolibdic acid-benzidine /PMA/B/ reaction were used to demonstrate histones in the nuclei. The amount of PMA-B pozitive spermatogonia and Leydig-cells in the testes and the granulosa cells in the ovaries of the different groups were compared.

Treatment with DES on the ninth day of incubation caused a decrease in the weight of the testes and an increase in the weigth of the ovaries. Treatment with AE did not affect the weight of testes but increased the weight of the ovaries. The amount of PMA-B positive spermatogonia was greatly decreased by DES and AE, while, the PMA-B positive Leydig-cells was not changed significantly. A single treatment with DES after hatching caused a 50% increase in the amount of PMA-B positive granulosa cells, while a double treatment /i.e. on the ninth day of incubation and after hatching/ provoked a 100% increase. AE caused no changes. The result shows the imprinting effect of DES in the fetal period. DES and AE inhibit the development and function of the male gonads while activates those in the females.

536 MELANOPHORE DIFFERENTIATION IN XENOPUS LAEVIS TADPOLES, SPECIAL REFERENCE TO DORSOVENTRAL PIGMENT PATTERN FORMATION.
Esther Kapa, L. Sárdy, Semmelweis University of Medicine, H-1445 Budapest, P.O.B. 370. Hungary

In Xenopus laevis tadpoles during the development after stage 26, a large number of dopa-positive cells were in the ventral region observed. Our present results proved that melanoblasts really existed in the ventral skin and showed their number, distribution pattern and degree of differentiation. The electron microscopic observations revealed that these cells became localised in the epidermal layer and they contained premelanosomes. In cultured skin-from ventral region-, fully matured melanophores appeared.

These results strongly suggest that a large number of melanoblasts are present in the ventral epidermis and they remain there without differentiation into melanized melanophores. Thus the positional difference of melanoblasts differentiation mainly contributes to dorsoventral pigment pattern formation of Xenopus laevis.

537 HEMOGLOBIN SWITCH AND OXIGEN TRANSPORT IN THE RED CELLS OF DEVELOPING CHICKEN EMBRYOS.
Ileana Arangi, Lanfranco Barberini, Carlo Cirotto. Institute of Cell Biology, Via Elce di Sotto, University of Perugia, 06100 Perugia, Italy.

The hemoglobin types produced in the red cells of developing chick embryo have been largely described. Two hemoglobin patterns appear during the embryonic life, one is typical of the early embryo and the other of the late embryo. Switching occurs at about 6 days of development and has been demonstrated to take place on a clonal base. The erythrocyte population of embryos up to 6 days of development (primitive line) substains the respiratory processes before the chorioallantois begins to differentiate. The function of the two erythroid lines, as well as the physiological meaning of their replacement, have been investigated in vivo by studying the effects of carbon monoxide on the survival of early embryos. The time course of the appearance of HbCO in particular areas of the embryo body, gives useful informations on the cycle time of the oxygenation-deoxygenation of primitive erythrocytes. A complete cycle takes about 15 min. By using this technique, we were able to show that early embryos (up to 6 days of incubation) can easily survive to long CO treatments. This finding suggests that the oxygen supply for cell metabolism is meanly provided by diffusional processes, until the hemoglobin switching takes place, and the chorioallantois appears. Diffusion must be considered also the major process involved in eliminating waste CO_2, in fact the red cells of early embryos lack carbonic anhydrase activity.

538 **HEPATOCYTE STRUCTURE OF THE TESTUDO HORSFIELDI TURTLE DEVELOPING EMBRYO**
A.A.Turdiyev, T.N.Volkova Institute of Biochemistry, Uzbek Academy of Sciences, Tashkent, USSR

Turtle embryo liver has been studied after 30, 50, 60 and 70 days of eggs incubation under the laboratory conditions. Reptile liver does not have lobe-like structure like that of mammals, but consists of repeatedly ramified cell beams braided with capillaries. Hepatocyte cytoplasma of 30 days old embryos contains some rounded mitochondria with light matrix and a few short crests. The cisterns of endoplasmic reticulum are mainly represented by smooth forms. The amount of bound and free ribosomes is insignificant. Glycogen granules and lipid drops are single. Further development leads to the sharp increase in the number of lipids that gradually fill up the whole cytoplasma and make it "foamy". Mitochondria matrix becomes dense and the amount of rough reticulum elements increases.

70 days old embryos have been shown to have enhanced levels of glycogen, its large granules fill up cytoplasma before hatching. By that time lipids tend to decrease in number.

Thus, the early stages of embryo development are characterized by the intensive lipid synthesis that is substituted by glycogen synthesis before hatching.

539 EFFECTS OF CYTIDINE ANALOGS ON ASCIDIAN EMBRYONIC DEVELOPMENT (Phallusia mamillata).
Egidio Puccia (1), MARGHERITA BRANNO (2), Luisa Tosi (2), Giuseppina Ortolani (1). (1) Istituto di Zoologia,Università di Palermo,90123 Palermo,(2) Stazione Zoologica, Villa Comunale, 80121 Napoli, Italy.

5-methylcytosine residues in DNA have been implicated to play a role in the control mechanisms that govern eukaryotic gene function and differentiation. Consequently, if specific patterns of cytosine methylation are associated with differentiation, it follows that compounds which alter these patterns would also interfere with the normal differentiation and development of the embryo. Analogs of cytosine, modified at position 5 of the pyrimidine ring, should be resistant to enzymatic methylation and hence might hamper normal gene expression and differentiation when incorporated into cellular DNA. We have studied the effect of 5-azacytidine (5-azaCR) in early embryogenesis P. mamillata. Eggs cultured in 10^{-4} M 5-azaCR developed into neurula-like stages, however still enveloped by their chorions. At the light microscope we noted three effects: a) the neural folds were not closed, although the larval sense organs were already formed: b) a shorter tail (6-10 chordal cells instead of 40): c) lack of mouvement, probably due to anamalous development of myotomes. These effects were overcome, in part, by adding cytidine or 5-methyldeossicytidine, simultaneously with the 5-azaCr. The results show that transient exposure to 5-azaCR causes an arrest in development except for the appearance of the sense organs.This arrest may be explained by the failure of new transcriptional events normally occuring after the neurula stage, that allow differentiation to proceed. It is also tempting to postulate that the new events might be, at least in part, mediated through site specific methylation and/or demethylation of a few cytosine residues on chromosomal genes.

540 *INFLUENCES OF BASAL DISC REGENERATION ON PATTERN FORMATION IN HYDRA ATTENUATA.*

MAHARAJAN, V.(1), DE PETROCELLIS, L.(2), MINEI, R.(2), ROSATO, F.(2). (1) CNR Institute of Molecular Embryology and (2) CNR Institute of Cybernetics, 80072 Arco Felice, Naples, Italy.

THE POSITIVE INFLUENCE OF BASAL DISC ON THE REGENERATION OF HYPOSTOME HAS BEEN RECOGNIZED AS AN IMPORTANT FACTOR IN THE PATTERN FORMATION IN HYDRA. HOWEVER DATA ON THE INFLUENCE OF BASAL DISC ON BUD FORMATION IN HYDRA ARE SCARCE. WE HAVE STUDIED IN HYDRA ATTENUATA THE INFLUENCE OF BASAL DISC ON BUDDING IN PRESENCE OF TUNICAMYCIN (TM) - A GLYCOPROTEIN INHIBITOR. IN HYDRA, REGENERATING BASAL DISC, TM CAUSES A DOSE DEPENDENT INCREASE OF BUDDING. INTERESTINGLY THE NUMBER AND LENGTH OF TENTACLES IN THE HEAD OF THESE ANIMALS ALSO INCREASED THOUGH THEY WERE NOT DECAPITATED.

NO SUCH INDUCTIONS WERE SEEN IN THE HYDRA REGENERATING BASAL DISC, BUT NOT TREATED WITH TM.

THESE RESULTS INDICATE THAT 1) GLYCOPROTEINS HAVE IMPORTANT ROLE IN PATTERN FORMATION IN HYDRA SUCH AS HEAD AND BUD FORMATION, 2) SOME GLYCOPROTEINS NEGATIVELY CONTROL PATTERN FORMATION AND 3) BASAL DISC INFLUENCES THE BUDDING PATTERN IN HYDRA.

541 ULTRASTRUCTURAL STUDIES ON PREVIOUSLY DRIED MAIZE POLLEN GRAINS ADHERED TO THE STIGMA.

Beáta Barnabás /1/, Katalin Liszt/2/, Loránd Fridvalszky/2/. /1/ Agricultural Research Institute, Hungarian Academy of Sciences, 2462 Martonvásár, and /2/ Department of Plant Anatomy, Eötvös Loránd University, 1088 Budapest, Muzeum krt. 4/a, Hungary

Dry Gramineae pollen grains require a longer period for adhesion to the stigma surface to initiate pollen tubes than fresh ones. We observed that in the case of previously dried Zea mays /line A 188/ pollen grains need a relatively longer lag-period for imbibition and adhesion. The aim of the present work was to investigate the ultrastructural changes and their cause during this lag-period. Artificial pollination was carried out using a pollen sample with 15% water content under semi-in vitro conditions and the pollinated stigma pieces were incubated for 10, 30 and 60 min. under staurated atmosphere. Material was prepared for TEM investigations by conventional ultramicrotechnical methods. Previously dried pollen grains required about 30 min. for dilatation and exudation as compared with the 5-10 min. at the controls. In addition to the observed ultrastructural changes in the pollen wall substructure and in the mobilization of starch, there were considerable changes in the amount of mitochondria and dictyosomes. Formation of numerous vesicles and the fusion of P-particles in the cytoplasm of the vegetative cell indicate increased metabolic activity preceding germination.

542 ON THE ULTRASTRUCTURE OF REGENERATING CELLS OF THE LIVERWORT RIELLA.

H. Lehmann, A. Apitius, Botanisches Institut der Tierärztlichen Hochschule, Bünteweg 17 d, 3000 Hannover 71, FRG

Non-meristematic single cells, two-cells systems and small tissue fragments, isolated from the thallus of Riella, regenerate completeplants (Lehmann 1966). At first the ultrastructure of regenerating single cells was studied. During the first two days of redifferentiation the cell wall is thickened by deposition of new wall material. Only then the cytoplasm increases and the cell gets more and more the appearance of a meristematic one. Cell division takes place about 12 days after isolation. Much more interesting is the regeneration process of an isolated two-cell system. Both cells start to regenerate, but usually only one cell divides and continues regeneration. During the process of redifferentation differences between the two cells must occur which lead to the result, that only one cell undergo division, while the other one is blocked in an special development stage.Stange (1977) proposed a hypothesis on cellular interactions in which she assumed, that one cell runs through the cell cycle faster than the other. The faster cell reaches the phase of auxin synthesis earlier and so gets a decisive projektion in redifferentation. We tried to correlate the physiological differences between the two regenerating cells with the fine structural ones. At first both cells thicken their cell wall, then the cytoplasm increases, small vacoules arise and the cytoplasm shows an increase in rough endoplasmic reticulum and polyribosomes. This occurs either in one or in both cells. Starch is accumulated in the chloroplasts of one or both cells, rarely in none cell. Usually a cell divides, if starch is present in the chloroplasts. In a cell, that is ready to divide, the cytoplasm is concentrated in the centre of this cell (systrophe), then cell division takes place.

Ref.: Lehmann, H.: Planta 71, 240 - 256 (1966)
Stange, L.: Planta 135, 289 - 295 (1977).

543 ULTRASTRUCTURAL CHARACTERISTICS OF SUBMERGED ACTIVE CLAVICEPS PASPALI STRAIN

Elizabeta Pertot(1), K. Jezernik(2), Matjana Didek-Brumec(3) and Helena Sočič(1). (1) "Boris Kidrič" Institute of Chemistry, 61000 Ljubljana, Hajdrihova 19., (2) Institute of Human Biology, Medical Faculty, 61000 Ljubljana, Lipičeva 2., and (3) LEK, Pharmaceutical and Chemical Works, 61000 Ljubljana, Celovška 135.

The main morphological characteristic of C. paspali strains producing large amounts of simple lysergic acid derivatives in submerged cultures is that they keep the filamentous structure in all the phases of growth and development. This significant feature is also a characteristic of our highly active C. paspali L-52 strain, which is described in this report.

Since no data have been available on the ultrastructure of C. paspali, we investigated the ultrastructural characteristics of our strain by using different methods of electron microscopy (EM).

Morphological characteristics of the vegetative and production mycelium established by scanning EM confirm the filamentous structure of the fungus in both phases of its development. By the freeze-fracturing method and transmission EM the fine structure of cells during development has been established and considerable differences in ultrastructure have been found between the vegetatitve and production mycelium. The cells of vegetative mycelium show homogenous cytoplasm with all subcellular components, while in the cells of production mycelium cytoplasm and organelles are desintegrated and in connection with this process several vacuoles arise. The number of lipid droplets in C. paspali L-52 is much smaller than in C. purpurea strains what probably could be connected with the excretion of secondary metabolites into the medium.

544 MYCELIAL AND ULTRASTRUCTURAL DIFFERENTIATION OF SAPROPHYTIC CLAVICEPS PURPUREA STRAINS

Matjana Didek-Brumec(1), Anka Puc(1), K.Jezernik(2) and Helena Sočič(3). (1)LEK, Pharmaceutical and Chemical Works,61000 Ljubljana, Celovška 135,(2) Institute of Human Biology, Medical Faculty, 61000 Ljubljana, Lipičeva 2, and(3) "Boris Kidrič" Institute of Chemistry, 61000 Ljubljana, Hajdrihova 19.

Changes in mycelial ultrastructure of two high producing saprophytic C.purpurea strains were followed: ergocornine-ergokryptine strain L-17 characterized by mycelial sporulation on solid medium and ergocristine producing non sporulating strain L-18.

Analyses of cell ultrastructure in vegetative and production phase of submerged fermentation were performed by electron microscopy(scanning and transmission) and the following characteristics were established:

1. Vegetative mycelium of both strains is filamentous. Cytoplasm includes all cell organelles among them also some vacuoles and few lipid bodies.
2. During the biosynthesis of alkaloids vegetative mycelium differentiates into sclerotia-like cells which are more expressed in L-17 strain. In the early stage of the production phase degradation of cytoplasm and cell organelles on the account of lipid bodies and vacuoles could be observed. Toward the end of the fermentation process two types of cells are differentiated. In one type of cells cytoplasm desintegrates saving only the inner cell membranes while in the other type of cells vacuoles fuse and cytoplasm remains intact. By comparing the strains, typical differences in number and shape of lipid bodies have been observed, these might be connected with accumulation of ergot alkaloids.

545 THE DYNAMICS AND CYTOLOGY OF STEROL ACCUMULATION IN THE YEAST Saccharomyces cerevisiae.

Božena Běhalová, Jana Svobodová, Josef Voříšek. Institute of Microbiology, Czechoslovak Academy of Sciences, Vídeňská 1083, CS-142 20 Praha 4, Czechoslovakia

In the yeast Saccharomyces cerevisiae the enhanced synthesis of sterols is induced by a nitrogen limitation in aerobic condition (low specific growth rate). Our analyses of yeast cultures during the induction of sterol biosynthesis showed a distinct decrease of protein contents and simultaneous increase in polysaccharide and lipid contents (expressed per units of dry weight). This was reflected also in the ultrastructure of cells where numerous lipid globules appeared around extensive vacuoles containing a lead-philic material. The sterols were localized in the lipid globules of intact yeast cells (spherosomes) by the fluorescence in UV-light.

546 FORMATION OF SYNAPTIC CONTACTS DURING NEUROONTOGENESIS.
G.G. Skibo, L.M. Koval. A.A. Bogomoletz Institute of Physiology, Academy of Sciences of the Ukrainian SSR, Bogomoletz str., 4, 252601 GSP, Kiev 24, USSR

Analysis of structural peculiarities underlying the formation of synaptic contacts which present a final stage of nerve cell development is one of the key problems in neurobiology. Dynamics of formation of different contacts between monolayer cultured cells of dissociated embryonal spinal cord and dorsal root ganglia has been studied by electron microscopy. Desmosome-like specialization of the membrane was shown to be an initial stage in the formation of both chemical contacts and gap junctions; later it combines with the above described contacts and as synapses matured desmosomes completely disappeared. In addition to usual synaptic contacts, complex glomerulilike structures of convergent and divergent types as well as contacts combining chemical and electrical components may be established. The latter contacts in vivo are typical of the spinal cord of lower animals.

547 CYTOLOGICAL EVIDENCES OF NEURAL CREST CELLS PARTICIPATION IN THE MORPHOGENESIS OF THE RAT PINEAL GLAND
V.Lačković, M.Japundžić, V.Bumbaširević, V.Čemerikić
Institute of Histology, Medical Faculty, Višegradska 26, 11000 Belgrade, Yugoslavia

The effects of gene mutation on the phenotipic characteristics of the pineal cells were studied in order to obtain additional data on the nature and embriological origin of distinct cellular subpopulations of the pineal gland. The investigation was carried out on the mutant strain of rats produced and elevated in our laboratory. Using different light and electron microscopic technics, we observed that the mutation affects morphogenetic behaviour of the melanocytic phenotypes, including production of typical melanosomes and tyrosinase activity. The mutant cells were most numerous in the peripheral area of the gland. With their long dendritic cytoplasmatic processes, containing typical melanosomes, they were closely aposed to the typical endocrine pinealocytes. It was concluded that deviant cells represent mutant forms of the connective tissue cells and/or of pinealocytes II, and that their precursors originate from the cephalic part of the neural crest.

548 EARLY SYNAPTOGENESIS IN CEREBRAL CORTEX IN HUMAN EMBRYOS.
A.A.Milokhin, A.M.Jakushova. Institute of Human Morphology, Moscow, USSR.

Embryonic synaptogenesis is the key process in the early brain formation, since with the apparence of the first intraneuronal connections, the long way of presumptive brain development into the system of functionally-active neuronal network, capable to the logical treatment of the new information, commence. In the present communication we announce new data on the embryonic synaptogenesis in cerebral cortex in human embryos. Study have been done by the methods of electron-microscopy. We found one of the main regularity in the individual human brain formation which shows that the formation of functionally competent interneuronal synaptic connections occurs very early in the prenatal ontogenesis. In the developing cerebral cortex in human embryos the first synapses appear to the end of the 7-th-the beginning of the 8-th weeks of the intrauterine development. In the presumptive human cerebral cortex it's marginal zone can be clearly seen up this time; from the ventricular layer into this zone the first young neurons of cerebral cortex - Cajal-Retzius cells - migrate; the first synaptic contacts are formed at soma and processes of these cells. Their structure completely corresponds to the pattern of functionally competent synapses. Data obtained suggest that already as early as in embryonic period of ontogenesis, the first neuronal mechanisms which are capable to the realization of the specific neuronal activity, are formed. This allows to imply that morpho-functional bases of the future highest neuronal functions in human beings have to be established very early as well - i.e. to the end of the 2 month of intrauterine life.

549 MORPHOLOGICAL CHANGES INDUCED BY BOTULINUM AND TETANUS TOXIN IN THE PRESYNAPTIC MEMBRANE OF PURE CHOLINERGIC SYNAPTOSOMES ISOLATED FROM TORPEDO MARMORATA.

G. Egea, J. Marsal, C. Solsona and J. Blasi. Dept. Histology and Cell Biology. School of Medicine. University of Barcelona. Hospital de Bellvitge. Barcelona. Spain.

Freeze fracture method exposes the membrane interior and it's has been extensively used to catch rapid structural changes in the presynaptic membrane in the course of the transmitter release. We used the sandwich freezing method applied to pure cholinergic synaptosomes to study the morphological changes induced by botulinum (BoTx) and tetanus (TeTx) neurotoxins during the transmitter release induced by 100mM KCl. This high concentration of external potassium produced a decrease in the IMPs (intramembranous particles) density at the P-face and an increase at the E-face in the presynaptic membrane of cholinergic synaptosomes. But when these synaptosomes were intoxicated by botulinum toxin, we observed that it inhibited the IMPs decrease at P-face and the E-face increase when they were stimulated by high concentration of potassium. This effect was reverted when the intoxicated synaptosomes were stimulated by A-23187 (20μM) in presence of high external calcium (10 mM). Tetanus toxin also produced a similar effect but didn't inhibit in a completely way ($p < 0.05$) like botulinum toxin.

550 EFFECT OF BOTULINUM TOXIN TYPE A ON THE ISOLATED NERVE TERMINALS OF TORPEDO MARMORATA

J. Marsal, C. Solsona, X. Rabasseda, J. Blasi, Departament d'Histologia i Biologia Cel.Tular, Fac. Medicina, Univ. Barcelona, Hospital de Bellvitge, Spain

ATP is co-stored with acetilcholine (ACh) into cholinergic synaptic vesicles. Both are co-released when cholinergic nerve terminals are depolarised, in a calcium dependent process. Botulinum toxin (Btx) type A inhibits specifically the depolarization-induced ACh release from cholinergic nerve terminals in both central and periferal nervous systems. Nevertheless, in brain cortical synaptosomes, ATP release was not significantly affected by Btx. Due to the heterogeneity of this preparation, it can be argued that ATP could proceed from non-cholinergic nerve terminals, where it is also stored with the neurotransmitter into synaptic vesicles. We have investigated the action of Btx type A in pure cholinergic synaptosomes isolated from Torpedo electric organ. Our results show that Btx type A inhibits specifically the K^+-induced release of ACh in a dose-dependent manner without affecting the release of ATP from cholinergic nerve terminals. Similar results are observed when different ACh release inductors are used (Gramicidin, Veratridine, A23187). We can prevent the Btx inhibitory action on ACh release by adding Rabbit antibotulinum toxin serum. Reversion of Btx action can be obtained by Ca^{2+}-uptake promote agents (Guanidine + 100 K, A23187) in presence of high external Ca^{2+} (10 mM), but we have seen that Btx does not modify the Ca^{2+} uptake into cholinergic synaptosomes. Two questions arise from this results. (i) Does all ATP released come from synaptic vesicles? (ii) Does cholinergic nerve terminals content only one kind of biochemically homogeneous synaptic vesicles?

551 **ELECTROKINETIC PROPERTIES OF ISOLATED RAT BRAIN MEMBRANE STRUCTURES- SYNAPTOSOMES.**

Svetla S.Stoylova,Department of Biophysics and Radiobiology,Biological Faculty, University of Sofia,Blv.D.Tzankov 8,1421 Sofia,Bulgaria

The electrokinetic properties of isolated rat forebrain synaptosomes have been investigated by the method of particle microelectrophoresis.The electrophoretic mobility has been measured as a function of pH and ionic strength of the suspending media and indicated that the synaptosomal surface contained weak acidic function.The surface of synaptosomes was determined to be negatively charged under physiological conditions.The isoelectric point has been observed at pH=4.0 The electrokinetic potential and the average surface charge density have been also calculated at physiological values of pH and ionic strength.The effect of monovalent-K^+,Na^+ and divalent-Ca^{2+},Mg^{2+} ions on the electrokinetic properties of synaptosomes was observed.The investigation of particle surface by the tchnique of microelectrophoresis can provide information about the biochemical environement of the synaptosomal surface and should be helpful in understanding more completely the structure of synaptosomal membrane and its reacting with a range of molecules.

552 ACETYLCHOLINE RELEASE INDUCED BY OUABAIN IN PURE CHOLINERGIC SYNAPTOSOMES

J. Blasi(1), V. Ceña(2), C. Gonzalez-García(2), C. Solsona(1), J. Marsal(1), (1) Departament d'Histologia i Biologia Cel.lular, Fac. Medicina, Univ. Barcelona, Hospital de Bellvitge (2) Departamento de Farmacología, Fac. Medicina, Univ. Autónoma de Madrid, Spain

The Na^+/K^+-ATPase is present in a wide variety of tissues, especially those that frequently need to restore the membrane potential. ^3H-ouabain-binding is a useful way to label Na^+/K^+-ATPase and can explain the possible effect of ouabain in basis to its affinity binding sites. Electric organ of Torpedo marmorata is a tissue that needs to expel large quantities of sodium ions after an electrical discharge. Therefore, it is covered by cholinergic nerve terminals on its ventral surfaces. Studing several subfractions of the electric organ we have observed that ^3H-ouabain-binding sites of high affinity (Kd = 25 nM) are mainly localized on the presynaptic membrane preparation. To intent to ascertain the possible relation of Na^+/K^+-ATPase in this subfraction and acetylcholine (ACh) release we have studied the effect of ouabain on isolated nerve endings from the electric organ. Such synaptosomes have shown to release ACh when different concentrations of ouabain ($10^{-3}M$-$10^{-9}M$) are placed in the incubation media. The amount of ACh release was dose-dependent and was not significantly altered when Ca^{2+}-free solutions were used, as described in previous works, in other preparations, by several authors. This can be explained if we assume that internal Ca^{2+}stores can be removed when intrasynaptosomal Na^+ concentrations arise, by inhibition of the Na^+/K^+-ATPase action as a sodium pump.

553 FINE STRUCTURAL CHANGES OF SYNAPSES IN THE SUPERIOR CERVICAL GANGLION OF ADULT RAT AFTER LONG-LASTING GABA ADMINISTRATION: A MORPHOMETRIC ANALYSIS

Ferenc Joó(1), László Siklós(1), Wendelin Dames(2), Joachim R. Wolff(2)
(1)Laboratory of Molecular Neurobiology, Institute of Biophysics, Biological Research Center, H-6701 Szeged POB 521, Hungary
(2)Department of Anatomy, University of Göttingen, Göttingen, West Germany

The superior cervical ganglion (SCG) of adult rats was exposed to GABA by long lasting microapplication. Serial sections were cut from the GABA-treated and the control ganglia, then the fine structure of individual synapses was investigated. The quantitative analysis of structural parameters of studied synapses showed that significant changes consisting of

- a reduction in size of presynaptic axon terminals,
- a decrease in the number of synaptic vesicles,
- a diminution in the extent of the postsynaptic membrane thickening at the dendritic side

developed on the effect of GABA-treatment. These results provide morphological evidence that long term application of GABA to the SCG exerts a combination of presynaptic and postsynaptic effects. It is suggested that the structural changes observed in the fine structure of individual synapses within the SCG may take part in the development of the synaptogenetic action of GABA.

554 AN OPTICAL MORPHOMETRICAL ANALYSIS OF THE SINGLY-INNERVATED NEUROMUSCULAR JUNCTIONS IN A NORMAL MUSCLE OF THE MATURE RAT.

E. Mayayo, R. Fenoll and J. Tomàs.
Departamento de Histología y Embriología General. Facultad de Medicina de Reus. Universidad de Barcelona. c/ Sant Llorenç, 21. Reus. Tarragona. Spain.

There is an increasing evidence mainly from the morphological point of view, that the neuromuscular sinapses are not rigid structures in the mature muscles of the adult animals, but in the contrary they are presumably submitted to a continous process of remodelling, so is, growth and retraction of the nerve terminals, and also degeneration and regeneration of certain endings.

We have performed a morphometrical study upon the silver impregnated (Bielschowsky-Gros method) motor nerve terminals in the singly-innervated synaptic areas of the sternocleidomastoid muscle cells of the adult normal rat (150 g.) specially for the parameters: terminal length (L), and number of branching points (BP) of the nerve endings. Because the high correlation between L and BP (r= 0.9), we have considered also the following indexes: the complexity index, CI=L.BP/100 and the "maturation" index, MI=BP.100/L.

There is a continous spectrum for both, CI and MI values that are not related with the size of the postsynaptic cell (muscle cell diameter).

Furthermore, the small MI-terminals are identical to those seen during sinaptogenesis, and the nerve endings with a higher MI value, shows an unbalanced situation for L and BP with an increase of BP and a diminution of L mean values, suggesting retraction phenomena occurring in a hiperbranched and "hipermature" terminals.

This data and the morphometric analysis performed in some synaptic areas receiving a nodal sprout of the parent axon together with the original ending, may suggest the existence of a remodelling process by substitution of the nerve terminals in a limited synaptic "space".

555 **MOLECULAR GENETICS OF THE ACETYLCHOLINE RECEPTOR**

DEVILLERS-THIERY, A. and CHANGEUX, J.P.
Unité de Neurobiologie Moléculaire
Institut Pasteur
25, rue du Dr. Roux, 75015 PARIS, France.

The nicotinic receptor at the neuromuscular junction and in the electric organ of certain fishes (Torpedo, Electrophorus) is an allosteric protein made of four different transmembrane glycosylated polypeptides in a stoichiometry $\alpha_2\ \beta\ \gamma\ \delta$. Acetylcholine binding sites are carried, at least in part, by the two α subunits, and all subunits participate to the formation of the ionic channel. cDNA clones for the four subunits have been isolated by molecular biology techniques for Torpedo, chicken and mammals. In view of the protein sequences, models of the polypeptides' transmembrane organisation have been proposed. Results will be presented on those models. Finally interaction in the synaptic membrane between the AchR and a cytoplasmic protein called v_1 will be discussed.

556 CORTICOSTRIATAL AND NIGROSTRIATAL PATHWAYS MODULATE THE ENDOGENOUS ACETYLCHOLINE RELEASE FROM THE NEOSTRIATUM.

J.Alberch, J.Blasi, J.Marsal, C. Solsona.Department of Histology and Cell Biology. Faculty of Medicine. University of Barcelona. Hospital de Bellvitge. Barcelona.Spain.

Corticostriatal and nigrostriatal pathways are the main inputs to the neostriatum. The aim of our work is to study the action of the transmitter of this pathway, glutamate and dopamine, on the intrastriatal cholinergics neurons, We used a chemiluminescent method to measure the acetylcholine release in the striatal slices. Our results show that glutamate and N methyl D Aspartate produce an evoked release of acetylcholine in a concentration of micromolar range; this effect is only reverted by NMDA receptor antagonists, but not by quisqualate receptor antagonists. Dopamine inhibit the release evoked by potassium, but not by aminoacids. We conclude that the modulation of acetylcholine release by excitators aminoacids and dopamine is produced through different ways.

557 INTRACEREBROVENTRICULAR OXYTOCIN MODIFIES NEURONAL-GLIAL AND SYNAPTIC RELATIONSHIPS IN THE ADULT HYPOTHALAMUS. C. Montagnèse, F. Rodriguez, D.A. Poulain, J.D. Vincent and D.T. Theodosis. INSERM, U.176, rue Camille Saint-Saëns, F-33077 BORDEAUX-Cedex, France.

Glial envelopment of neurons and the establishment of synaptic circuitry are thought to occur during development and then to remain unchanged under normal conditions. However, in the supraoptic (SON) and paraventricular nuclei of the adult hypothalamus both these parameters are modifiable, under physiological stimuli. Upon stimulation of the oxytocin-secreting system, glial coverage of oxytocin neurones diminishes and contiguous cell bodies and dendrites become directly juxtaposed ; the neurones also receive an increased synaptic input, from terminals that contact two adjacent elements simultaneously (Theodosis et al., 1986, Neuroscience, in press). The modifications are reversible and glial reinsertion occurs once the system is no longer activated. In order to see whether oxytocin itself is responsible for these anatomical changes, we studied the effects of intracerebroventricular administration of oxytocin upon the structure of the SON. Using miniosmotic pumps delivering fluid at 0.5 µl/h, synthetic oxytocin, vasopressin (2 µg/ml) or vehicle (artificial CSF) were infused continuously into the 3rd ventricle of non-lactating rats for 7 days. Ultrastructural analyses of the SON indicated that infusion of oxytocin altered the morphology of the nucleus to an extent similar to that observed after normal stimulation (e.g. lactation) : close to 45% of all neurosecretory cell bodies were directly apposed to each other or to adjacent dendrites, as compared to 10% in the controls (vasopressin- or vehicle-infused rats). The proportion of cell bodies sharing the same synapse also increased only in the SON of animals infused with oxytocin (from 6 to 14%). Immunocytochemical analysis showed that the anatomical changes affected almost exclusively oxytocinergic neurones and not adjacent vasopressin cells. Our observations suggest that the neuroanatomical changes are under hormonal control and provide evidence that a neuropeptide can act centrally to modify the interrelationship of particular neurones and their surrounding glial cells.

558 EFFECTS OF VINCRISTINE (VC) AND VINBLASTINE (VB) ON THE PORPHYRIN SYNTHESIS OF PRIMER NEURAL TISSUE CULTURES. Irene Durkó, Anna Juhász. Department of Neurology and Psychiatry. Medical University, H-6701 Szeged, P.O.Box 397, Hungary

Neurotoxicity studies to date relating to VC and VB have emphasized their antimitotic action and their disturbing effects on the axonal transport in connection with neurofilament accumulation. In view of the close interaction of the cyclic nucleotides via the adenyl-cyclase systems on porphyrin synthesis, and the assumption that VC and VB are linked equally specifically to the GTP receptors (1,2), we have begun experiments on their effects on porphyrin synthesis in primary neural tissue cultures. The cultures were prepared from 7-14-day-old chick embryo brain by mechanical dissociation in MEM-Eagle medium in the presence of 20% foetal calf serum (3). The porphyrin products formed from 185 KBq/ml tritiated delta-aminolaevulinic acid in 24 hours were isolated by thin-layer chromatography and expressed as percentages. VC and VB in conc. of 10^{-7}M were found to increase the total porphyrin production in glia and neurocyte cultures. Besides the morphological changes, a considerable porphyrin synthesis enhancement can be observed in the presence of either VC or VB.

Among other points, the extent to which the accumulated porphyrin products are responsible for the development of the irreversible neuropathies observed during their therapeutic application remains to be clarified. 1) R.W.Weisenberg, Studies on the Chemistry of Microtubule Protein. Ph.Dissertation.Univ.of Chicago (1968), 2) R.W.Berry, M.L.Shelanski, J.Mol.Biol.71, 71-80 (1972), 3) I.Durkó,A.Juhász, in "Neuropeptides and Psychosomatic Processes" edits.E.Endrőczi et al.Acad.Press (1983) pp.751-757.

559 DISTRIBUTION OF CALCINEURIN IN THE CENTRAL NERVOUS SYSTEM OF THE RAT STUDIED BY LIGHT MICROSCOPY IMMUNOCYTOCHEMISTRY. M. Solimena[1], C. Klee[2], M.H. Krinks[2] and P. De Camilli[1]; 1) Dept. of Med. Pharm. and CNR Center of Cytopharm. Univ. of Milano; 2) NCI, NIH, Bethesda, Maryland.

Calcineurin is a Ca^{++}/calmodulin-stimulated protein phosphatase highly concentrated in brain which appears to be the brain form of phosphatase 2B. It has a Mr of 80,000 and it is composed of two subunits of Mr 60,000 and 15,000, respectively. In the present study we have investigated by light microscopy immunocytochemistry the distribution of calcineurin in the rat CNS using a polyclonal antibody directed against the holoenzyme. Calcineurin immunoreactivity was found to have a widespread distribution but remarkable regional variations in staining intensity and pattern of immunostain were seen. In all regions immunoreactivity was detectable only in neurons and inside individual neurons it was homogeneously present in perikarya dendrites, axons and axon terminals. Nerve cells containing the highest concentration of immunoreactivity included cells of the caudate projecting to the globus pallidum and the substantia nigra (cells of the dorso-medial quadrant of the caudate being more heavily labeled than cells of the venrolateral quadrant), pyramidal cells of layer II, III and VI of the neocortex, CA2 and CA1 pyramidal neurons of the hyppocampal cortex. As a result of this uneven distribution of calcineurin, the pattern of immunostaining resembled a stain for perikarya and dendrites in regions where the local neuronal population contained high levels of calcineurin (for example, in the caudate) and a stain for nerve terminals in regions where calcineurin-poor cells were innervated by calcineurin-rich cells (for example, in the substantia nigra). One of the best in vitro substrates for calcineurin is the phosphoprotein inhibitor DARPP-32. DARPP-32 is highly concentrated in the cells of the caudate that project to the globus pallidum and the substantia nigra, (Ouimet et al., J. Neurosci. 4, 111, 1984) i.e., cells which contain high levels of calcineurin. In addition, like calcineurin, DARPP-32 is homogeneously distributed in the cytoplasm of these cells. Thus, DARPP-32 might be a physiological substrate for calcineurin.

560 ION-DEPENDENT PEPTIDASE/S/ ACTIVITY AGAINST SUBSTANCE P ANALOGUE'S FRAGMENT: /^{125}I-TYR$_8$//PYROGLUTAMYL$_5$/ SP$_{5-11}$ IN SUBCELLULAR FRACTIONS FROM DIFFERENT AREAS OF RAT BRAIN. W.A. Turski, Lilla Lachowicz, II Dept.of Biochemistry, Institute of Physiology and Biochemistry, School of Medicine, ul. Lindleya 6, 90-131 ŁÓDŹ, Poland.

Peptidases might be involved both in degradative "death" or "birth" of peptide hormones. The aim of this paper was to study degradation of TP/the peptide specified in the title/ in the following areas of rat brain: 1/cortex,2/hippocampus, 3/striatum,4/thalamus and hypothalamus,5/cerebellum and 6/medulla oblongata. The subcellular fractions: nuclear, mitochondrial, synaptosomal, myelin-rich, lysosomal-microsomal/L/, cytosolic-microsomal were incubated with TP in three kinds of media: I/similar in ionic composition to the intracellular fluids/I/, II/similar to the extracellular fluids/E/, III/Tris-EDTA sucrose medium. Next, the ion-exchange scheme of separation of undegraded TP and its degradation products was applied. The biggest total and specific activity/SA/can be shown in hippocampus area. There are mostly "middle" products, some N-terminal and occasionally C-terminal. The biggest SA/usually in L fraction/occurs in most cases in I-medium - rich in K^+ and Mg^{2+} ions; still, one can find undegraded TP in the cell interior after incubation of brain a-reas in E-medium. Then, the nuclear is the place where a lot of label /both degraded and-or-undegraded/ can be found.

561 SUBCELLULAR LOCALIZATION OF AN ENDOGENOUS LECTIN IN RAT CEREBELLA

Dragana D.Svrzić,* Sladić, d.,** Dujić A.,*** Radojčić*** M., Ćuperlović M.,***** Levental**** M., Rakić Lj.*

* Institute of Biochemistry, Medical Faculty, Belgrade
** Department of Chemistry, Faculty of Science, Belgrade
*** Institute of Experimental Medicine,Military Medical Academy, Belgrade
**** Institute for Biological Research, Belgrade
***** INEP, Zemun

Lecetin with mannose specificity was isolated from microsomal (Ms) and mitochondrial (Mh) membranes of rat cerebella. Antibodies were obtained by immunizing rabbits with this lectin previously bound to ovalbumin. These antibodies were purified and labeled with fluoresceine isothyocyanate. This antibody was used for indirect fluorescence staining of the original lectin incorporated in Ms and Mh membrane pallet preparations.

562 TWO TYPES OF GECKO VISUAL PIGMENTS DISTINGUISHED BY IMMUNOCYTOCHEMISTRY.

Ágoston Szél and P. Röhlich. 2nd Dept. of Anatomy, Histology and Embryology and Lab. I of Electron Microscopy, Semmelweis University of Medicine, Budapest. H-1450, Tüzoltó u. 58. Budapest, Hungary.

On tangential sections of the gecko retina, the following photoreceptor cell types can be distinguished; single cone type A, double cone types B and C, all of them organized in a linear pattern. Microspectrophotometric studies show that the accessory member of C double cones contain a blue-sensitive pigment, while all the other elements contain a green-sensitive one. Monoclonal antibody COS-1 (anti-cone visual pigment antibody raised against a chicken visual pigment) and anti-rhodopsin (polyclonal anti-bovine opsin produced in rats) were used to discriminate the visual cells in the nocturnal gecko, Teratoscincus scincus.

By light microscopic immunocytochemistry of tangential semithin sections of the gecko retina, the blue-sensitive cone outer segment (accessory member of double cone type C) proved to be positive with the anti-rhodopsin antibody. In contrast, all the cone types containing a green pigment (single cones A, both members of double cone type B and the principal member of double cone type C) bound the COS-1 antibody. To our knowledge, this finding is the first incidence when cone pigments were characterized by both their spectral and immunologic properties. By electron microscopic immunocytochemistry the ultrastructure of the anti-rhodopsin-positive outer segments was different from that of the others in that it had a loose, poorly preserved appearance. Another finding was that the rarely occurring triplet cones contained one middle member with a loose structure giving a positive anti-rhodopsin reaction and two lateral (principal) members which bound the COS-1 antibody.

563 THE HETEROGENEITY OF PHOTORECEPTORS IN THE CHICKEN RETINA AS REVEALED BY ANTI-VISUAL PIGMENT ANTIBODIES.

Ágoston Szél and P. Röhlich. 2nd Dept. of Anatomy, Histology and Embryology and Lab. I of Electron Microscopy, Semmelweis University of Medicine, Budapest. H-1450, Tüzoltó u. 58. Budapest, Hungary.

Microspectrophotometric and electrophysiologic studies show that cone outer segments of vertebrate retinas contain several types of visual pigments with different absorption maxima. While rhodopsin, the rod visual pigment is biochemically characterized, the molecular properties of the cone visual pigments are unknown.

Three different anti-visual pigment antibodies were produced previously in our laboratory; COS-1 and OS-2 are monoclonal antibodies raised against visual pigments from chicken photoreceptor membranes. AO is a polyclonal anti-bovine opsin serum produced in rats.

By light microscopic immunocytochemistry of frozen semithin sections and immunoblotting of chicken photoreceptor membrane suspension, three kinds of visual pigments could be localized in the chicken photoreceptor cells. COS-1 labelled a visual pigment (mol.w.: 33 kD) localized in the outer segments of both members of the double cones and the single cones having a red oil droplet. AO antibody stained rhodopsin (mol.w.: 35-37 kD) present in the rod outer segments and, additionally, in two types of single cones (with deep yellow and yellowish-green oil droplets). OS-2 stained all the above mentioned outer segments together with the single cones having a colourless oil droplet. This cone type which does not bind either COS-1 or AO may represent a third, immunologically distinguishable class of cones.

564 ELECTRON MICROSCOPE OBSERVATIONS ON THE ULTRASTRUCTURE OF OOCYTE OF AMPHIOXUS (BRANCHIOSTOMA BELCHERI GRAY)

Wang Dehai, Wang Deyao (Ouang Teyio). Institute of Cell Biology, Xiamen University, Xiamen, Fujian, P.R.C.

By the freeze-cutting scanning and freeze-etching electron microscopes, we observe three layers membranes of oocyte in Amphioxus, vitelline membrane, secondary membrane and jelly coat. Some mamillation-like microvilli were separately distributed on the surface of jelly coat. The microphotographs of scanning electron microscope proved that the microvilli are closely arranged on the surface of vitelline membrane in branched shape. Under transmission electron microscope, we observed mircopinocytotic vesicles that were produced by vitelline membrane can be developed into yolk granules. The RER and Golgi complex can be developed into yolk granules. The amount of mitochondria gradually increases with the development of oocyte. They distribute in the area of periphery of nucleus, RER and Golgi complex. Only part of mitochondria can be also developed into the yolk granules. Phagocytosing activity of lysosomes appearing in period of vitellogenesis was observed. Under freeze-etching electron microscope, we observed the ultrastructure of nuclear pore complex that consists of the annular granules, the periphery granules and the central granules. This ultrastructure identify the model structure of nuclear pore complex. Our result proves that the ultrastructure of oocyte of Amphioxus is fundamentally similar to the oocyte of certain invertebrate and vertebrate.

565 COMPARTMENTALIZATION OF K,Ca,Mg,S and P IN INSECT OMMATIDIA.

Irina Burovina, Felix Gribakin, Kyrill Ukhanov. Sechenov Institute of Evolutionary Physiology and Biochemistry, Academy of Sciences of the USSR, 194223 Leningrad, Thorez pr.44, USSR

Intracellular content of Ca,K,Mg and that of S and P was studied by X-ray microanalysis. Cryosectioning and embedding in epoxy-resin following freeze-drying were used for all the elements to be preserved in situ. Concentration of K in different cells and compartments of the compound eye of cricket Acheta domesticus L. widely ranged from 50 to 260 mM/kg wet mass with the highest one measured in pigmented zones. These zones were characterized by high concentrations of Na (60 mM) and Ca (up to 190 mM). Both K and Na, not Ca, were easily washed off by water from the sections of embedded tissue. Pigment granules in photoreceptors and pigment cells of the cricket eye are considered to be the main intracellular ion reservoir. In the eye of honey bee Apis mellifera L. high concentration of Ca was observed only in certain pigmented zones of ommatidia. Probably high Mg, not Ca, is stored by primary pigment cells. Studies of Ca and Mg content in the honey bee eye colour mutant snow were aimed to clarify if non-pigmented eyes were capable of ion sequestring. Ca content in snow eyes was found to be the same as that in the eyes of normal bees (+/+) whereas the level of Mg and S proved to decrease dramatically both in photoreceptors and pigment cells. Different Ca/Mg and K/Na ratios are known to be inherent in pigmented zones of various tissues in insects and vertebrates which differ in S/P ratio as well. The reason for a certain chemical element to dominate quantitatively is not clear as yet.

566 STRUCTURAL DIFFERENCES IN THE PHOTORECEPTORS OF MALE AND FEMALE INDIVIDUALS OF THE BALTIC SEA AMPHIPOD DULICHIA PORRECTA (CRUSTACEA, PERACARIDA) .

V.Benno Meyer-Rochow (1), Helmuth Stephan (2). (1) Department of Biological Sciences, University of Waikato, Hamilton, New Zealand, and (2) Zoologisches Institut, Christian-Albrechts-Universität, D-23 Kiel, Federal Republic of Germany.

In the Baltic Sea amphipod Dulichia porrecta male and female individuals posses eyes that differ in size and organization. The long axes of the very slightly elliptical eyes of adult male and female individuals measure 500-600 um and 250 - 300 um, respectively. Ommatidial numbers also vary and in male and female specimens amount to 77 and 89, respectively. Ommatidial diameters are larger in males (32-34 um) than in females (28-30 um). Internally, the main difference is the somewhat broader (2.7 vs 2.4 um) and longer (40 vs 32-35 um) rhabdom in male animals and the significantly greater amount of screening pigment granules in the eyes of females. Anatomical features shared between males and females consist of bipartite crystalline cones, five retinula cells per ommatidium, a perirhabdomal space as well as high numbers of reflecting vesicles. Ultrastructural derangements of rhabdom or microvilli following several hours of exposure to very bright light were of a minor nature. On anatomical grounds alone, we conclude that males have eyes which should possess very high absolute light - sensitivity whereas for females resolution and visual acuity would appear to be at premium.

567 ELECTRON MICROSCOPIC IMMUNOCYTOCHEMISTRY OF THE RAT THYROID: LOCALIZATION OF THYROGLOBULIN AND THYROID HORMONES. Pyra Ring and Viktor Johanson, Dept. of Anatomy, University of Göteborg, Sweden

In thyroid follicle cells newly synthesized thyroglobulin (TG) in exocytic vesicles is secreted into the follicle lumen. There, TG is iodinated and the thyroid hormones (T_3, T_4) formed. TG is taken into the cell by endocytosis and hydrolyzed, and the thyroid hormones are liberated.

A postembedding, EM-immunocytochemical technique for localization of TG, T_3 and T_4 in the rat thyroid was developed. Protein A-colloidal gold and IgG-colloidal gold were chosen as markers because of high sensitivity and resolution and suitability for quantification. Different procedures of fixation, embedding and processing were examined in order to obtain optimal immunolabeling and preservation.

With anti-TG, good labeling was obtained on both Epon and etched LR White sections. Labeling was very dense over the colloid, macropinocytic vesicles and exocytic vesicles, less dense over RER and Golgi, and very scanty over nuclei and interstitium. With anti-T_3 and anti-T_4 a moderate labeling was seen on Epon and etched LR White sections. The labeling was mainly located over follicle lumens and macropinocytic vesicles, but some cytoplasmic label was seen. The gold particle density over different colloids had a coefficient of variance of about 25% for both anti-TG and anti-T_4. The density of label was three times higher when 10 nm gold particles were coupled to IgG than to protein A.

TG immunoreactivity was well preserved in fixation solutions containing up to 1% glutaraldehyde and 4% paraformaldehyde but was slightly less at 2.5% glutaraldehyde. OsO_4 up to 0.6% usually did not reduce labeling. T_3 and T_4 showed immunoreactivity after fixation with solutions of 4% paraformaldehyde and 2% paraformaldehyde + 1% glutaraldehyde. Thyroid hormones were more sensitive to osmium fixation than TG, and were only occasionally immunoreactive after 0.6% OsO_4 fixation.

The present study shows that by using defined conditions of fixation and embedding, both TG and thyroid hormones can be localized with the gold immunolabeling technique.

568 EXPERIMENTAL, ULTRASTRUCTURAL STUDIES ON HUMAN THYROID TISSUE

Johan Mölne (1), Viktor Johanson (1), Erik Jörtsö (2), Mikael Nilsson (1), Torsten Öfverholm (1), Staffan Smeds (2), Lars E. Ericson (1). (1) Dept. of Anatomy, University of Göteborg, (2) Dept. of Surgery, University of Linköping, Sweden

Human thyroid tissue can be transplanted to nude, athymic mice with retained functional and morphological properties. The transplants are rapidly vascularized by vessels from the host.

Pieces (about 10 mg) of normal and pathological human thyroid tissue were inserted subcutaneously in each groin of the nude mouse. The activity of the transplants was monitored by external counting of ^{125}I. The transplants were fixed by vascular perfusion.

TSH acutely stimulated exocytosis as well as colloid endocytosis. Long-term stimulation by thyroid stimulating immunoglobulins, prepared from patients with diffuse, toxic goiter, induced growth (increased ^{3}H-thymidine incorporation) and increased the follicle cell volume about 2.5 times. The stimulated transplanted tissue was also characterized by the appearance of colloid vacuoles, typical for hyperfunctioning human thyroid tissue. The vacuoles were surrounded by membrane fragments presumably derived from the apical plasma membrane by membrane shedding. Thyroglobulin was present in the follicle lumen as well as in intracellular locations (colloid droplets, vesicles) as revealed by electron microscopic immunocytochemistry (protein A-gold). Thyroperoxidase, the key enzyme in synthesis of thyroid hormones, occurred in cytoplasmic locations as well as in the apical plasma membrane as shown by electron microscopic cytochemistry. Peroxidase in the apical plasma membrane is probably the enzyme active in iodination as electron microscopic autoradiography short times (5 min-2 h) after injection of ^{125}I showed that protein-bound label was almost completely located extracellularly in the follicle lumen.

The present observations show that transplantation to nude mice provides a system for experimental observations on human thyroid tissue not otherwise possible.

569 STUDIES IN CORONAL LAYER OF ODONTOBLASTS IN THE HUMAN DENTAL PULP BY SCANNING ELECTRON MICROSCOPE

Herbert Wegner (1), Hartmut Schneider (2). (1) Policlinic of Conservative Dentistry Karl-Marx-University, GDR-7010 Leipzig, Nürnberger Str. 57, and (2) Centre of Electr. Microscopy Karl-Marx-University, Sect. of Biol. Sciences, GDR-7010 Leipzig, Talstr. 33

Coronal layer of odontoblasts from human dental pulp was stuied in the scanning electron microscope. The purpose of this investigation was to receive more detailed knowledge in the formation of odontoblast layer. Twenty-four permanent teeth with no caries were removed from young patients for orthodontic reasons. Immediately after extraction, the teeth were stored in 8 per cent solution of neutral formalin. The complete pulpal strudures were obtained by fracturing the teeth carefully into halves. The samples were dehydrated by alcohol or aceton, dried and coated with gold.

Specimens of normal teeth showed in the coronal part of the pulp chamber numerous dentinal tubules on the predentinal surface and some of them contained the TOMES fibres. The predentin demonstrated a bushy feltwork of dentin matrix fibres.

In a smaller number of präparations odontoblast cells were frequently seen. Here and there the odontoblasts were covered in a network of so-called von KORFF fibres (Figures).

570 THE STUDY OF SOME ENDOTHELIAL ANTIGENS BY MONOCLONAL ANTIBODY. Dan Predescu-Mandas, Institute of Cellular Biology and Pathology, Bucharest - 79691, Romania

Endothelial cells (EC) isolated according to the method of Simionescu and Simionescu (Microvasc.Res. 16: 426-452, 1978) were used to immunize BALB/c mice. Splenocytes from immunized mice were fused with SpAg20/14 murine myeloma cells following the procedure of I.Mellman (Yale University, personal communication). A ratio of spleen cells to myeloma cells of 10:1 in the presence of polyethyleneglycol 4000 (Merck) as a fusogen was used. The growing hybrids were selected in hypoxanthine-aminopterin-thymidine (HAT) medium for two weeks. At the end of this period, the hybrids that recognized the antigens from the EC surface - by modified ELISA assay - were selected. Out of 80 hybrids which secreted antibodies that reacted with endothelial antigens, one was subject to cloning according to R.P.Siraganian et al. (Methods in Enzymology 92E: 17-27, 1983). Four clones that secreted McAb recognizing endothelial antigens - by ELISA modified assay - were thereafter selected. Two clones were tested by indirect immunofluorescence on frozen sections of rabbit heart, lung, kidney and aorta - to establish the antibody specificity. As second antibody, goat anti-mouse serum coupled with FITC was used (Capell). Both selected monoclonal antibodies reacted specifically with large vessel endothelial cells of rabbit heart, lung, kidney, and aorta;other types of cells were not stained. The occurrence of similar antigens on the endothelial cells of microvasculature could not be ruled out. The indirect immunofluorescence on the bovine aortic EC in culture shows a uniform distribution over the endothelial cells. The immunoelectron microscopy performed on the same cells shows a random distribution of antigen over the EC plasmalemma. Control experiments in which the monoclonal antibody was omitted gave negative results. Immunoprecipitation followed by SDS-PAGE reveals a precipitation band of about 70,000 D for one McAb and another one of about 100,000 D for the second McAb. (Supported by Ministry of Education, Romania, and by NIH Grant HL-26343).

571 CONTRIBUTION TO THE GIANT MULTINUCLEATE CELL FORMATION

Karel Smetana, jr.
Institute of Anatomy, Faculty of Medicine, Charles University, U nemocnice 3, CS 128 00, Prague, Czechoslovakia

The knowledge of the in vivo cell and implant interaction is necessary for the succesfull implantation of an artificial material in the clinical praxis. A fusion of specialized macrophages to the giant multinucleate cell is a characteristic feature of the reaction against the implant as a foreign body. The subcutaneous implantation of the celophan foil in experimental rats induces the formation of 3 types of giant cells: Langhans cells /type 1/, cells with nuclei in the center of the cytoplasm /type 2/, widely extended cells with clumping of enlarged nuclei in the cytoplasm /type 3/. Type 1 and 2 apparently represent developmental stages of type 3. The positivity of histochemical reaction for hydrolases and oxidoreductases is lower in the type 3 then in the type 1 and 2. Type 3 cells cover a large area of the implant surface and form an active boundary between the implanted foil and surrounding tissues. The reaction to hydrogel foils appeared to be limited with respect to the formation of giant cells.

572 CHARACTERIZATION OF MASTCELLSUBTYPES OF THE RAT BY MONOCLONAL ANTIBODIES.

N. Rossow(1), H. Repke(1), S.B. Sâvoly(1), J. Odarjuk(1), L. Karawajew(2). (1) Institute of Drug Research, Academy of Sciences of the GDR, 1136 Berlin, Alfred Kowalke Str. 4, and (2) Central Institute of Molecularbiology

Mice were immunized with a mixture of acetonextracted and Glutaraldehyde treated rat peritoneal mast cells and a granule free membrane preparation of these cells. We established four hydromas which secrete monoclonal antibodies (mabs) reacting with a surface determinant of rat peritoneal mast cells.

These four mabs did not react with white blood cells, thymocytes, spleen cells, bone marrow cells, lymph node cells and brain and liver tissue. There is a certain fraction of peritoneal marcrophages which can also be detected by the four mabs.

The mabs permit the detection of subtypes among the peritoneal mast cells which were previously unknown. Furthermore, quantitative differences could be observed between mesenterial mast cells and pleural mast cells. Lung mast cells do not seem to exhibit the corresponding antigen. the mabs do not react with rat IgE, the IgE Fc-recptor and are not IgE itself. They do not interfere with 6 different modes of histamine release from the mast cell.

573 FACTOR XIII OF BLOOD COAGULATION IN HUMAN MONOCYTES AND MACROPHAGES.
Róza Ádány(1), J. Polgár(1), G. Szegedi(2), Z. Nemes(3), Mária Kávai(2), L. Muszbek(1). (1) Department of Clinical Chemistry, (2) IIIrd Department of Medicine, and (3)Department of Pathology, University School of Medicine, H-4012 Debrecen POB 40. Hungary

The presence of Factor XIII subunit a was demonstrated in human monocytes by immunoperoxidase staining using specific antisera against factor XIII and its subunits. This finding was verified by immunobiochemical techniques, as well. In an immunoblotting system after SDS polyacrylamide gel electrophoresis of denatured monocyte homogenate a protein band comigrating with Factor XIII subunit a showed positive reaction with antibodies against this subunit or whole Factor XIII. In contrast, no subunit b of Factor XIII could be detected by either of these methods in monocytes. Activity measurements were carried out by the dansylcadaverine incorporation assay in the absence and presence of anti-Factor XIII antibody with and without thrombin activation. The expression of transglutaminase activity required thrombin and was completely abolished in presence of anti-Factor XIII antibody, which clearly indicate that practically all the transglutaminase activity measured in monocytes comes from Factor XIII. FXIII was retained during differentiation of monocytes into peritoneal macrophages. In cytoplasm of the latter cell type immumostaining for FXIII subunit a was especially intensive in adhesive pseupods and around phagocytic vacuoles. Subunit a of FXIII is also present in tumor associated macrophages involving lymph nodes with Hodgkin's disease.

Factor XIII of monocytes and macrophages might have a role in formation of stable fibrin at the site of inflammation or around tumor cells and may be involved in various cellular functions, as well.

574 WEIBEL-PALADE BODIES IN PIG MEGAKARYOCYTES.
Jeannine GEBRANE-YOUNES (1°, Ludovic DROUET (2), Jacques-Philippe CAEN (2), Louis ORCEL (1). (1) Service Central d'Anatomie et Cytologie Pathologiques, Faculté de Médecine St-Antoine, 27 rue Chaligny F 75571 PARIS CEDEX 12, France, and (2) Département d'Angio-Hématologie, Unité INSERM U 150, Hôpital Lariboisière, 6 rue Guy Patin F 75010 PARIS, France.

Originally described in vascular endothelial cells Weibel-Palade bodies are specific of this cellular type, as they have never been reported elsewhere. Weibel-Palade bodies serve as storage granules for various proteins and specifically for von Willebrand factor, which is stored in microtubular form. Besides endothelial cells, von Willebrand factor is also synthesized by bone marrow megakaryocytes. Von Willebrand factor has been located in α- granules of megakaryocytes and blood platelets. Using transmission electron microscope, we describe true Weibel-Palade bodies in pig megakaryocytes, and also structures characterized by microtubules located only at their periphery. We designate these structures as intermediate structures. Von Willebrand factor is most likely stored in microtubular form in these two types of structures. This assumption is supported by the absence of microtubules in these granules in cells obtained from pig homozygous for the von Willebrand disease (lacking totally this protein). We hypothetize that during bone marrow maturation of megakaryocytes, Weibel-Palade bodies are the original storage structure for von Willebrand factor and mature to intermediate structures and then to α- granules, where the von Willebrand factor is associated with other proteins.

575 EFFECT OF EMETINE ON THE THYMIC CELL POPULATIONS.
Oláh, I.(1), F.Antoni(2), N.G.Luat(2), Ildikó Csuka(2),and G.Bánfalvi(2). (1) 2nd Department of Anatomy,Semmelweis University Medical School,H-1450 Budapest. and (2) 1st Institute of Biochemistry,Semmelweis University Medical School,H-1444 Budapest, POB 260. Hungary

Thymic hormon(s) produced by thymic epithelial cells are regulatory proteins influencing the T cell maturation. Effect of emetine which is a potent protein synthesis inhibitor was studied on the thymus. A single dose of emetine administrated subcutaneously to mice decreased the thymus weight which reached the lowest level by day three of treatment. At this time the histological studies revealed that the thymic cortex was deeply involved in the T cell depletion while the medulla did not show any valuable changes. The cortical area was filled with macrophage-like cells containing large number of phagosomes. The absolut number of thymic adherent cells was much higher than that of the control animals. The presence of the high number of adherent cells may indicate that the emetine changes the surface character of certain thymic cell population and/or adherent cells migrate to the thymus. These possibilities are under investigation. No anti-SRBC anti-bodies (IgM+IgG) were found by hemagglutination test.

Protein, RNA and DNA syntheis was measured by incorporation of isotop labeled precursors which indicated a substancial loss by 24 hours after emetine administration. Decrease in the protein, RNA and DNA syntheis was 60%,40% and 90%, respectively. By day seven of emetine treatment the thymus both histologically and biochemically returned to normal.

576 LOCALIZATION OF DIFFERENT ANTI-KERATINS ANTIBODIES IN NORMAL AND IN DOWN'S HUMAN THYMUSES.
Mariangela Aita(1),Ada Amantea(2),Vincenza Indelicato(1),Claudia Gandin(1). (1) Institute of Human Physiology, Faculty of Medicine, University La Sapienza, p.le A.Moro 5, 00185 Rome and (2) Institute of Dermatological Hospital San Gallicano, 00153 Rome, Italy.

It is widely accepted that epithelial cells of the thymus are responsible for the secretion of the thymic hormonal factors, needed for the differentiation of the thymocytes. Epithelial cells contain keratins that differ with various epithelia in their origin and are produced at different steps during their differentiation. Three biopsy specimens of thymuses from normal children, 2,19 and 24 months old, and two specimens from children with Down's syndrome, 18 and 36 months old, were obtained during heart surgery. Five µm thick paraffin sections were stained by peroxidase-anti-peroxidase method using a polyclonal anti-total keratin serum, a monoclonal anti-keratin B that normally reacts with the epidermal supra-basal strata, a monoclonal anti-keratin C, that normally reacts with all squamous and ductal epithelia (kits from Ortho Diagnostic). The immuno-reactivity to anti-total and to anti-keratin C was present in the cytoplasm of epithelial cells localized in the medulla and in Hassall's corpuscles. The immuno-reactivity to anti-keratin B was present in the epithelial cells of the subcapsular zone,the cortex, the medulla and in Hassall's corpuscles. No significant difference was noted as far as the distribution and the intensity between normal and Down's thymuses are concerned.

The results of our research seem to indicate that both in normal and in Down's thymic epithelial cells there are various steps of differentiation, supported by the presence of different keratins,this could be correlated with different secretory roles of these epithelial cells.

577 THE PROPORTION OF M CELLS IN THE FOLLICLE-ASSOCIATED EPITHELIUM IS NOT INFLUENCED BY THE DEPLETION OF INTRAEPITHELIAL LYMPHOID CELLS.
Piotr Siciński(1), Jerzy B. Warchoł(2), Wojciech Bem(1) and Jan Rowiński(1). (1) Department of Histology and Embryology, Medical School, Chałubińskiego 5, 02-004 Warsaw, Poland, and (2) Department of Histology and Embryology, Medical School, Święcickiego 6, 60-781 Poznań, Poland.

M cells are antigen sampling cells which occur in the follicle-associated epithelium (FAE) covering Peyer's patches of the small intestine, but are not present in the villus epithelium. Several authors hypothetized that M cells arise from absorptive cells of the FAE under the influence of the contact with intraepithelial lymphoid cells (IEL) which are abundant in the FAE. We have tested the above hypothesis by morphometric methods.

Using transmission electron microscopy we have determined the frequency of the occurence of M cells and IEL in the FAE of conventionally housed mice and in the FAE of mice kept under specified pathogen-free (spf) conditions.

It was demonstrated that the frequency of M cells in the FAE of mice kept in spf conditions did not differ significantly from that in conventional mice (9% and 11%, respectively), while the ratio of the number of IEL to the number of epithelial cells of the FAE was 2 times lower in mice kept in spf conditions as compared to conventional mice (0.35 and 0.66, respectively, the two ratios being significantly different).

Our results were interpreted as inconsistent with the hypothesis that M cells differentiate as a result of the contact of FAE absorptive cells with IEL.

578 FINE STRUCTURES OF EARTHWORM HEART
S.G. Pal and P.K. Dinda. Dept. of Zoology, University of Calcutta, Calcutta-700019, India

The heart muscles of Pheretima posthuma possess two kinds of myofilaments: thick and thin, measuring respectively as 27-33 nm and 6-7 nm in diameter. The ratio between the two filaments is 1:16. A-, L- and Z-bands are clearly observed in the TEM. Z-bodies (0.2 - 0.3 µ in length) formed of thin myofilaments, take an oblique course along the muscle fibre. Numerous flattened tubular profiles peculiarly alternate with the Z-bodies along the muscle fibre. Several mitochondrial bag evaginate on the surface of the muscle cells. The peripheral non-contractile sarcoplasm shows several non-extensive tubules, vesicles and vacuoles. Attachment plaques infrequently populate the sarcolemma. The presence of cytoplasmic patches containing elementary granules in the motor interneurons is observed in the heart wall of earthworm. These granules resemble neuronal secretory products. These results provide unusual information for the invertebrate cardiology.

579 CELLULAR SYSTEMS INVOLVED IN WATER ABSORPTION BY EVERSIBLE VESICLES IN ARCHAEOGNATHA (INSECTA).
František Weyda. Institute of Entomology, Czechoslovak Academy of Sciences, Branišovská 31, 37005 České Budějovice, Czechoslovakia

Eversible vesicles are paired organs situated on the ventral side of body segments in many groups of arthropods. At rest they are retracted in the body cavity, whereas in functional state they are fully everted. They have an important role in water economy in these animals because their main function is to absorb water from wet substrates (soil, stones, wood etc.). An eversible vesicle contains several cellular systems directly or indirectly participating in water uptake.

The eversible vesicle is divided into two parts differing in their morphology and function: a non-absorbing part with epithelium resembling the hypodermis of other body parts, and an absorbing part with a specialized transporting epithelium (apical microvilli, deep infoldings of basal plasma membrane associated with numerous mitochondria). There is also a difference in the structure of the cuticle of each part of the eversible vesicle. The absorbing part of the eversible vesicle has a well developed layer of subcuticle (which is very thin in the non-absorbing part) containing polysaccharides which participate in the absorption of water by the transporting epithelium. Several highly specialized sensilla (cuticular part is lacking) are distributed within the transporting epithelium: their function is to inform the central nervous system whether water is being absorbed by the respective vesicle. The lumen of an everted vesicle is divided by vesicular diaphragm (formed by connective tissue) enabling circulation of hemolymph during absorption.

580 **UNEVEN DISTRIBUTION OF THE CILIATES DILEPTUS ANSER IN THE GRADIENT OF GAMONES EXCRETED BY CELLS OF A COMPLEMENTARY MATING TYPE.** **A.L.Yudin, S.Ju.Afon'kin. Institute of Cytology, Academy of Sciences of the USSR, 194064 Leningrad, 4 Tikhoretsky Avenue, U.S.S.R.**

The Dileptus anser cells belonging to any of the three mating types revealed so far in this ciliate, are known as autonomous excreters of gamones (Tavrovskaya, 1974, 1979). The gamone concentration gradient was established in the agar chamber. The procedure was as follows. Two cylindrical depressions were made in 2% agar I mm apart; cells from the clone to be tested were put into one of the depressions; cell-free fluid from a culture of a complementary mating type or cells from the culture were introduced into the other. In any case, 1-1.5 hrs after the onset of the experiment cells under test accumulated near the agar partition between two depressions i.e. within the zone with the highest concentration of a heterologous gamone. Characteristically, the cell movement slowed down; homotypical conjugating pairs were formed and conjugational cell divisions were observed by the end of the 6-hour experiment. This effect was registered with every possible heterologous (with respect to mating type) combinations of clones (three clones of different mating type were used), but was absent when homologous clones interacted (three clones of each mating type were taken for this control series). No matter what the mechanism of the ciliate response to heterologous gamones may be (it may taxis, or kynesis, or something else), it evidently enables the complementary cells to approach each other which is necessary for their subsequent conjugational interactions.

581 PHOTOSENSITIVITY OF EUGLENA GRACILIS AND RELATED SPECIES. Leszek Kuźnicki, Ewa Mikołajczyk. Nencki Institute of Experimental Biology, 02-093 Warsaw, 3 Pasteur, Poland

Euglena gracilis responds to an increase of light intensity(the step-up photophobic response), and to a decrease of light intensity(step-down photophobic response). The permanently streptomycin-bleached Euglena gracilis, colorless flagellates:Astasia longa and Peranema trichophorum - exhibit the step-up response, but not step-down. The decrease of the light intensity threshold for the step-up response as a result of change of the external medium is observed in both E.gracilis and A.longa. Therefore, this phenomenon is not dependend on the presence of stigma or paraflagellar swelling (PFB). The decrease of the threshold for the step-up response in E.gracilis is associated with a partial or complete inhibition of the step-down response. As soon as the flagellates recover the ability to perform the step-down response, the threshold for the step-up reaction increases. On the basis of recently obtained results(1-3) it is clear that the membrane on the emergent flagellum is an important structure for the step-up response in colorless and green flagellates. We also postulate that the threshold for the step-up response determines the ability of Euglena gracilis cells to express the step-down response.
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582 A POSSIBLE ROLE OF TETRAHYMENA CELL MEMBRANE DNA IN HORMONAL IMPRINTING
Zs. Darvas, V. László, P. Kovács and G. Csaba, Semmelweis University of Medicine, H-1445. Budapest, P.O.B. 370. Hungary

The first encounter between a target cell and a hormone results in hormonal imprinting at several levels of phylogenesis. The phenomenon had been observed in the unicellular organism Tetrahymena pyriformis, too.

In insulin /10^{-6}M/ pretreated Tetrahymena cells the FITC labeled insulin binding capacity has been increased. The influence of imprinting had been demonstrated after 48 h /in the 16th generation/ thus there is reason to postulate a fixing of imprinting either at gene or membrane level.

There are some evidences for the presence of DNA in cell membrane even in Tetrahymena cells, too. Supposing the role of this membrane DNA in the hormonal imprinting, Tetrahymena cells had been treated with DN-ase I for 5 and 10 minutes, before or after insulin imprinting.

In DN-ase pretreated cells imprinting did not develop and DN-ase treatment after imprinting abolished the effect of insulin. The experiments call the attention to the possible role of membrane DNA in the fixation of hormonal imprinting.

583 A FREEZE-ETCH STUDY OF SUGAR-TOLERANT YEASTS
A. Svoboda (1), T. Török (2). (1) Dep. Biol., Purkyné University, Brno, CSSR, (2) Dep. Microbiol., University of Horticulture, Budapest, Hungary

Sugar-tolerant yeasts have been known for more than 100 years. In recent years there has been considerable interest in their physiology and fine structure, in the mechanism and molecular biology of their tolerance or requirement for high solute concentration.

Physiological studies have shown that sugar-tolerancy in yeasts can be explained by mainly non-metabolic regulation processes taking place at the level of the plasmamembrane.

Ultrastructural studies of sugar-tolerant yeast cells and protoplasts did not reveal any particular differences to non-tolerant species except abudant periplasmic bodies thought to be connected with localization and activity of invertase.

In our experimental approach using freeze-etching technique we tried to answer the following emerging questions:

(i) What is the ultrastructure of plasmamembrane in sugar-tolerant cells like?
(ii) Are there differences in the ultrastructure of two sugar-tolerant yeast species differing in their sugar-tolerancy?
(iii) Are there some particular ultrastructural features in sugar-tolerant cells in comparison with non-tolerant ones?

584 GENETICAL APPROACH TO OXYGEN TOXICITY. Tomasz Biliński and Jadwiga Litwińska. Zamość College of Agriculture, Agricultural Academy of Lublin 22-400 Zamość ul. H.Sawickiej 102 Poland

The role of intermediate products of dioxygen reduction in cytotoxic effects ascribed to oxygen molecules was studied in vivo using various yeast mutants with changed response to oxygen stress. It has been documented that superoxide radical exerts its deleterious effects on yeast cells directly and the role of other oxygen species derived from it is hardly detectable /1/. Yeast Saccharomyces cerevisiae however cannot be considered as a typical eukaryotic organism due to its inability of synthesizing polyunsaturated fatty acids /pufa/. These fatty acids are known as main target molecules during oxidative stress and their peroxidation leads to cytotoxic effects. As fatty acid content could be easily manipulated in yeast this organism was used to evaluate the contribution of pufa peroxidation process to the cytotoxic effects of oxygen. Results obtained show, that yeast cells containing linolenic acid as the main constituent of phospholipids are hypersensitive to oxygen stress and therefore the dominant role of pufa peroxidation in oxygen toxicity finds experimental support in vivo. Yeast cells not supplemented with pufa were used to study alternative processes leading to cell death as a consequence of oxidative stress. The role of various deleterious processes initiated by oxygen and presumptive mechanisms protecting against them will be discussed.

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585 RELATIONSHIPS BETWEEN CELL GROWTH AND MITOCHONDRIAL FUNCTION IN CANDIDA : ANTIMITOTIC EFFECT OF SOME ANTIBIOTICS AND CENTRAL NERVOUS SYSTEMS DRUGS.

Nelson Marmiroli (1), PierPaolo Puglisi (1), Anna Salsi (1), Nadia Artoni (2), Massimo Melissari (2), Giorgio Truzzi (2), Filomeno Tedeschi (2). (1)Institute of Genetics and Department of Pathology - University of Parma 43100 PR Italy

Dyes which inhibit mitochondrial translation (ethidium bromide, berenil, acriflavine) and some drugs active on the mammalian central nervous system (CNS) (imprimine, amitriptiline chlorimipramine, chlorpromazine, promazine) are able to arrest, at wery low concentration, the process of mitotic reproduction of both pathogenic and not-pathogenic Candida species. Biochemical and ultrastructural evidences showed that some of these drugs inhibited mitochondrial respiration, the formation of some respiratory enzymes and the biogenesis or the assembling of several mitochondrial components. One and two-dimensional electrophoretic analysis of total proteins from cells labelled "in vivo" in the presence of these drugs showed the disappearance of some polypeptides. Fractionation of the "in vivo" labeled cells into the various subcellular components (mitochondria, membranes, cytosol)have evidenced that these polypeptides belong to the mitochondria and are also probably translated within the organelles. Restriction-enzymes analysis of the mitochondrial DNA prepared from cells grow with these drugs failed to evidence significant changes in its physical structure. In eucaryotic organisms some drugs, as the dyes and the CNS drugs described here, can therefore arrest mitotic division through the inhibition of the mitochondrial function.

586 CHANGES IN THE PHOTOSYNTHETIC ACTIVITY OF UNICELLULAR GREEN ALGA CHLORELLA PYRENOIDOSA WITH A MODIFIED MEMBRANE STRUCTURE.

G.Gyulai/1/, E.Lehoczki/2/ /1/ Department of Plant Breeding,University of Agricultural Sciences,H-2103 Gödöllő, Páter Károly ut 1., Hungary, and /2/ Department of Biophysics, József Attila University, H-6722 Szeged, Egyetem utca 2., Hungary.

The function of photosynthetic membrane was studied on changed membrane structure of Chlorella pyrenoidosa caused by Metronidazole /2-methyl-5-nitroimidazole-1-ethanol/. This compound affects strongly both the desaturation processes of fatty acids and the photosynthetic electron transport chain by its electron acceptor ability. Metronidazole was applied in sublethal concentration with a 72-hour period of treatment in alga culture. The structural changes resulting from Metronidazole application were confirmed by electron microscop, and low temperature fluorescence tests. The changes of the membrane lipid composition during treatment was characterized a 30 % reduction in total fatty acid content, and an accumulation of 16:3 unsaturated fatty acid. Due to direct structural changes distinctive changes occured in the function of the Second Photosystem /PS II./. At low light intensity the Light Intensity Dependent Oxygen Induction showed a more effective activity than the control. This result suggested an accelerated action of electron transport chain. With active Oxygen Induction and a 25 % Equilibrium Oxygen Evolution distinctive fluorescence induction activity was observed. On the bases of these results the interpretation of the Fluorescence Induction Curve for testing has to be re-evaluated.

587 MEMORIAL LECTURE: VERZÁR'S IDEAS ON THE AGE-DEPENDENT PROTEIN CROSS-LINKING IN THE LIGHT OF THE PRESENT KNOWLEDGE. I. Zs.-Nagy, F.Verzár International Laboratory for Experimental Gerontology (VILEG), Hungarian Section, University Medical School, H-4012 DEBRECEN, Hungary

Verzár was one of the pioneers of the experimental gerontology in the fifties. He formulated the basic concept according to which the reasons for biosenescence should be searched in macromolecular alterations.The main experimental proof supporting this concept was the age-dependent alteration of the behavior of collagen in heat- or salt-denaturation assays. Verzár (1956) interpreted those changes by assuming that a number of covalent intra- and intermolecular cross-links come into being in the collagen during the life, stabilizing the molecular structure of the tendons in both mechanical and chemical sense. Due to the extremely low turnover rate of collagen, these alterations accumulate and we can measure the consequences of them in the experiments mentioned above.

It should be stressed that Verzár was aware of the possibility that other proteins may also be subject to similar alterations: he and his coworkers performed experiments on the chromatin and revealed that chromatin proteins may be cross-linked during aging as well. Already Verzár mentioned in his papers and books that one of the possible reasons for the cross-link formation may be the effect of the oxygen free radicals proposed by Harman (1956) as general causes of the age-dependent deterioration of the living functions. Unfortunately, the biochemists of 30 years ago were very skeptic about this interpretation.

The preparative biochemistry has delivered convincing proofs already during the seventies showing that the basic concept of Verzár was correct: in the collagen various chemically identified cross-links became known. On the other hand, the research in the field of oxy-radicals developed tremendously during the last decade and hard proofs are available demonstrating that the cross-linking of proteins and other organic molecules are due mostly to the effect of oxygen free radicals like superoxide and hydroxyl free radicals. This latter one is formed in the living systems mainly by the reaction of hydrogen peroxide (a standard product of all aerobic systems) with the transient metals like iron. Electron spin resonance spectroscopic evidence is available showing that practically all amino acids, and proteins are very sensitive toward hydroxyl free radical induced damage (Zs.-Nagy and Floyd, 1984), and the reason for which the living systems must replace all their components rather frequently during their life is the extremely high reaction rate of these damaging processes. If we consider that the extent of the free radical induced damage depends on the density of the local structural environment of the macromolecules, one can obtain a very useful guide for the understanding of the processes of cellular aging.

The merit of Verzár cannot be overestimated, if we consider that in the early fifties the molecular biology was just starting and he was a physiologist who had the courage to formulate new ideas even against the contemporary general knowledge. His example teaches us also today to be open minded in the interpretation of the experimental data and to follow the rationale of the nature, if we want to understand something about the very complicated process called life.

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588 AGING, VITAMIN E DEFICIENCY AND ELECTROLYTES IN RAT BRAIN CORTICAL CELLS.

C. Pieri, C. Giuli, C. Bertoni-Freddari Center of Cytology, Gerontological Research Department I.N.R.C.A., Via Birarelli 8, 60121 Ancona Italy.

Intracellular electrolyte content of large brain cortical cells during aging as well as in adult rats fed with a vitamin E deficient diet has been investigated by energy dispersive X-ray microanalysis. The in vivo Rb^+ uptake and release of the same cells in vitamin E deficiency was also studied. The main results are as follows:

1) In old and in vitamin E deficient animals the total monovalent electrolyte content increased when compared to the young ones.

2) Vitamin E deficient rats accumulate more Rb^+ than the age matched normally fed animals. The Rb^+ discrimination ratios, calculated on the basis of Rb^+ and K^+ contents of both cortical cell cytoplasm and cerebrospinal fluid, are higher in vitamin E deficient rats than in the controls, showing the same pattern as the old animals (Gyenes et al. 1984).

Our results support the view that an enhanced lipid peroxidation is a common factor responsible for the impaired membrane functionality both during aging and vitamin E deficiency.

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589 CELLULAR AGING - INTRACELLULAR WATER AND IONIC SHIFTS
Thomas von Zglinicki[1], Gy. Lustyik[2].(1) Dept.of Electron Microscopy, Institute of Pathology, Charité, GDR-104 Berlin, Schumannstr.20-21, and (2)V.I.L.E.G., University Medical School, H-4012 Debrecen, Hungary

During the aging of rats the water concentration (WC) in hepatocytes and myocytes decreases to 95±1% of the adult value. As measured by X-ray microanalysis of frozen-dried cryosections, the decrease is insignificantly in nuclei and RER of hepatocytes and significantly in the cytoplasm. A preferential decrease of the WC has been found in mitochondria. The WC in old (24 months) rats amounts to 76±6% of the adult (6 months) value in hepatocytes and to 84±4 % in myocytic mitochondria. Ionic concentrations per compartment water in the myofibrils of myocytes and in the cytoplasm, RER, and regions of decondensed chromatin within the nuclei of hepatocytes do not change with age. However, there is an increase in the concentration of Na^+, K^+, and Cl^- in the regions of condensed chromatin (including the actively transcribed genes) together with an increase in the P concentration, i.e. the nucleotide/protein ratio. Most interestingly, the ionic strength in mitochondria increases with age to 142±25% of the adult value in hepatocytic mitochondria and to 253±63 % in myocytic ones, mostly due to an increase in the K^+ concentration. This might serve to stabilize the mitochondrial membrane potential despite of increasing membrane stiffness with age. However, increasing ionic strength will lead to condensation of polyelectrolytes and therefore adversely affect the function of mitochondrial enzymes. Without assuming structural or functional alterations of the mitochondrial enzyme system in vitro, this mechanism could explain the decreased energetic potential of aged cells.

590 AGE-DEPENDENT ALTERATIONS OF THE INTRACELLULAR WATER AND ELECTROLYTE CONTENT OF HEART AND MUSCLE CELLS.
Gy. Lustyik, F. Verzár International Laboratory for Experimental Gerontology (VILEG), Hungarian Section, University Medical School, H-4012 DEBRECEN, Hungary

Our recent measurements demonstrated a significant increase of the electrolyte concentrations in brain and liver cells of old animals as compared to the young ones. According to the membrane hypothesis of aging this increase of the intracellular ionic strength results in a general condensation of the cytoplasmic colloid, slowing down the rate of the cellular protein synthesis in postmitotic cells.

Using energy dispersive X-ray microanalysis of bulk specimens, intracellular concentrations of monovalent ions (Na^+, K^+ and Cl^-) were measured in heart and muscle cells, i.e in other postmitotic cells of 1-25 month-old rats. Separate measurements were performed in order to get the elemental concentrations in the dry mass of the cells, and to determine the intracellular water and dry-mass content. The in vivo concentrations were calculated from these two measurements supposing that the monovalent ions are dissolved in the cell water.

The results of analyses indicated that similar tendency of the age dependent alterations were found in the heart and muscle cells as demonstrated previously in neurones and hepatocytes. A statistically significant decrease was measured in the water content of the cells, suggesting an increase of the density and viscosity of the cytoplasmic colloid during aging. The loss of the cellular water was accompanied by a significant increase in both the single ion concentrations and the total monovalent ion content of the intracellular water.

591 MORPHOLOGICAL ASPECTS OF SYNAPTIC PLASTICITY IN THE AGING BRAIN.
C. Bertoni-Freddari, C. Giuli, D. Paci, C. Pieri. Center of Cytology, Gerontological Research Department I.N.R.C.A., Via Birarelli 8, 60121 Ancona Italy.

A quantitative investigation was carried out on the age-related morphological changes of the synaptic junctions in the cerebellar glomeruli and in the supragranular layer of the hippocampal dentate gyrus of young (3 months) adult (12 months) and old (30 months) rats. The numerical (Nv) and the surface (Sv) density as well as the average length ($\bar{L}$) of E-PTA stained synapses was calculated by means of morphometric methods. The results we obtained showed a similar trend in both the areas of the CNS studied. Nv significantly increased between young and adult rats and significantly decreased in the old group when compared both to young and adult animals. Sv appeared to be unchanged in the young and adult group of animals, whereas it was significantly reduced in the 30-month old group. $\bar{L}$ showed a decrease between 3 and 12 months of age and appeared to be markedly increased in the old animals when compared to adult values.

From the present findings it can be inferred that number (Nv) and size ($\bar{L}$) of the synapses are in a close inverse relationship which, through the organism's lifespan, aims to maintain the constancy of the surface area (Sv) among the dendritic network. By considering Nv, $\bar{L}$ and Sv altogether per age group we were able to obtain a reliable measurement of the morphological aspect of synaptic plasticity through different periods of the life. With regard to aging we found that, despite the "compensative synaptogenesis" brought about by the increased size ($\bar{L}$) of the synaptic appositions, the reactive capacity of old nerve cells is seriously impaired.

592 EFFECTS OF AGING ON THE RECYCLING VIA THE PENTOSE CYCLE AND ON THE KINETICS OF GLYCOGEN AND PROTEIN METABOLISM IN VARIOUS ORGANS OF THE RAT. Niedermüller,H. Department of Gerontology, Institute of Physiology, University of Veterinary Medicine, Linke Bahngasse 11, A-1030 Vienna, Austria

The rate of metabolic kinetics and the frequency of biological cycles may be correlated with the rate of aging and the maximum life span potential. Therefore investigations either into aging changes of such parameters within one species or into differences between species may give some information about the genetic programming of the aging process. Male Sprague-Dawley rats aged 3, 9, 12, 16, 24 and 33 months (m) were used to determine the age changes of those metabolic pathways mentioned in the title, using the liver (LI), kidney (KI), heart (HE) and the brain (BR). The maximum percentage of glucose utilization via the pentose pathway, compared to the total glucose utilization, was calculated after intravenous (i.v.) administration of D-[1-^{14}C]-, D-[6-^{14}C]- and D-[U-^{14}C]-glucose by the determination of the trioses (as lipids) 3 h after the application. The rate of the glucose utilization was determined likewise 10, 20, 30, 60, 90 and 120 min after the application. Glycogen kinetics was determined analogously. Total protein metabolism was followed using the essential amino acid L-[2,5-^{3}H]-histidine. The results indicate a decrease of the glucose utilization via the pentose pathway in relation to glycolysis and a reduction of the rate of this pathway in the investigated organs, a small but not significant change of the kinetics of glycogen metabolism (a lower turnover rate) in LI, KI and HE, and a reduced rate of protein metabolism in the investigated organs during the aging process. The reduction of the pentose pathway may possibly be the cause of higher lipofuscine accumulation in the cells of HE and BR, lacking sufficient reduction equivalents for lipid metabolism. Furthermore there could exist a connection with the reduced protein turnover, because less riboses are provided for the synthesis of nucleic acids. Finally it is not possible until now to decide if these effects are accompanying phenomena or genuine causes of aging, but we get some insights into their counterdependence.

593 CELLULAR AGING OF THE RETICULOENDOTHELIAL SYSTEM.
D.L. Knook, M.A. Horan, A. Brouwer. TNO Institute for Experimental Gerontology, P.O. Box 5815, 2280 HV Rijswijk, The Netherlands.

The reticuloendothelial system (RES) consists of macrophages and other endocytic cells which contribute to natural resistance to disease agents. The RES has been implicated as being partly responsible for the observed age-related decrease in resistance to pathogenic microorganisms in man and experimental animals. It is largely unknown to what extent age-related deficiencies in specific functions of reticuloendothelial cells contribute to the decrease of RES functions. This study describes experiments focused on Kupffer and endothelial cells of the liver which represent a major part of the RES and are primarily responsible for clearance of potentially harmful substances from the circularion.

The age-related changes in the clearance of an RES test colloid (heat-aggregated colloidal albumin (CA)), and of E.coli endotoxin were investigated using female BN/BiRij rats of various age groups. With CA, studies were performed on the plasma clearance and on the endocytic capacity of whole liver and of Kupffer and endothelial cells in vivo, as well as on the kinetics of endocytosis by Kupffer cells in culture. The results showed that the plasma disappearance of endotoxin was significantly prolonged in rats of 24 and 36 months resulting in an increase in half-life of about 50% as compared to young rats. The plasma clearance of colloidal albumin, the uptake by the liver and the endocytosis by endothelial cells were unchanged with age. In contrast, an age-related decrease in endocytic capacity was observed for Kupffer cells in vivo and for Kupffer cells which were isolated from rats of various ages and maintained in culture. It is concluded that, although the overall capacity of the liver to take up some test substances might not be affected in old age, there is a decrease in Kupffer cell endocytosis. Since a number of potentially harmful agents, such as endotoxins, are exclusively cleared from the circulation by Kupffer cells, a decreased endocytic capacity of these cells may be an important factor in the increased sensitivity to these agents during aging.

594 IMPAIRED ADAPTIVE RECEPTOR REGULATION AND AGEING

L. Piantanelli, Centre of Biochemistry, Gerontol. Res. Dept. - I.N.R.C.A., Ancona, Italy.

Many neuroendocrine functions are altered in old animals and their study may represent important steps in the understanding of the mechanisms of ageing. A deeper insight, however, can be achieved by investigating the responsiveness to stimuli, which may reveal alterations not evident in the unstimulated conditions. At this level of study, many of such impairments have been found to be caused by receptor changes. In the present paper a third level of study is suggested in order to gain evidence of some remote failure of adaptive processes strictly linked to intimate mechanisms of ageing. As, at the second level of study different receptor characteristics can frequently be found at the basis of age-related alterations of biological responsiveness, at the proposed third level altered capacity of receptor regulation may be hypothesized as responsible for altered cell adaptation following hormone and drug stimuli. Experimental data are given which support this view. Beta-adrenergic receptor regulation has been studied in animal and human cells following hormone and drug injection. Impaired receptor regulation has been found in mouse submandibular glands and brain cortex and in human lymphocytes following injection with thyroid hormones and beta-blockers. The age-related trend of some alteration is tentatively compared with the kinetics of ageing parameters as predicted by a mathematical model of mortality of populations.

595 MONOAMINES AND THEIR METABOLITES IN DIFFERENT AREAS OF THE CENTRAL NERVOUS SYSTEM DURING POST-NATAL DEVELOPMENT AND AGEING.A.Machado,J.Cano and M.Santiago.Dpto.Bioquimica de la Facultad de Farmacia Universidad de Sevilla.

The biogenic amines serotonin (5-HT),noradrenaline (NA),and dopamine (DA) have an important role in neurobiology,since it is assumed that they have a function as neurotransmitter.We have developed a method by means of HPLC in conjunction with an electrochemical detection which was used to determine the monoamine and their metabolites Tryptophan (Trp),5-hydroxytryptophan (5-HTP),5-hydroxyindolacetic acid(5-HIAA) 5-hydroxytryptophol,normetanephrine (MNM),metanephrine (MN),3-methoxytyramine (3-MT),3-4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in different areas of the CNS of the rat.

We present a study of the concentrations of the biogenic monoamines in the visual system (lateral geniculate,superior colliculus,posterior thalamus and visual cortex) and in the striatum of the rat.Our result show that in all structures of the visual system there is an increase in the content of 5-HT,5-HIAA DA,DOPAC,NMN,MN during postnatal development and ageing.However,NA was increase in the geniculate nucleus and visual cortex. On the other hand,in the lateral posterior thalamus and the superior colliculus,it undergoes a decrease until 3 months. Thereafter,NA concentration increases until 24 months. Other metabolites remaing without statiscally significant changes until 24 months. Only the Trp underwent a decrease in all structures. The importance of the monoamines in the striatum is illustrated by the changes that have been found in diseases as Huntington's and Parkinson's. At the same time,the increase of these diseases with the ageing has been described. Our results show that the striatum has high levels of DA and its metabolites,.which reached higher levels at 6 months. Thereafter,they underwent a decrease during the ageing . Among the metabolites of DA,the DOPAC was the metabolite found in major concentration. NA concentrations were about 3-fold less than DA ,being the Trp the metabolite found in higher levels.

596 INFLUENCE OF ENVIRONMENTAL STRESS ON LIPOFUSCIN PRODUCTION

Eugenia Aloj Totaro(1), Francesco A. Pisanti(1), Vincenzo Cuomo (2). (1)Chair of Biology and General Zoology, I Medical School, University of Naples, V.le M.Cristina, 18 I-80122 Napoli, Italy and (2) Res. Lab. CIBA-GEIGY, Torre Annunziata (Napoli), Italy.

Because many heavy metals damage biological structures by way of the production of free radicals, the age pigments seems able to be considered also as markers of environmental stress.

In the Torpedo marmorata CNS the electric lobes show the presence of large quantities of age pigments. The morphology of the pigment granulations is typical: finely granular in younger animals, and grossly granular and digitate in older animals. Attempting to discover the conditions that favour the production and accumulation of age pigments derived from the lipoperoxidative effect induced by free radicals, we evaluated the defense mechanisms against free radical damage present in the torpedo central nervous system. We found: absence of vitamin E, low levels of glutathione, absence of 5-thiohistidine, low levels of superoxide dimutase (SOD) and glutathione peroxidase, and absence of glutathione reductase.

In animals exposed to different levels of iron, copper and lead pollution, we have found a direct relationship between the quantity of age pigments present in the electric lobe and the level of pollution in the animal's environment.

We have obtained same results in fungi exposed to several heavy metals action.

597 Age dependent Erythrocyte (RBC)-Phagocytosis of the isolated rat liver after galactosamine-hepatitis and alpha-naphthyl-isothiocyanate cholestasis

T. Vömel and D. Platt, Institute of Gerontology, University of Erlangen-Nuremberg, FRG

Homologues rat RBC suspended in an isotonic buffered saline, free of immunoglobulines were perfused through the isolated perfused rat liver. Galactosamine (GalN) hepatitis and Alpha-naphtyl-isothiocyanate (ANIT) cholestasis were used for experimental hepatosis. Phagocytosis of RBC, the levels of GOT, GPT, Potassium and the Redoxquotients of Lactate/Pyruvate and ß-OH-Butyrate/Acetoacetate were determined.

Results: 1. There were 2 different patterns in the GalN injured group, given the same dosis. The heavily damaged group (maximal GOT, GPT) showed a significantly higher phagocytosis of RBC than the moderately damaged livers.

2. The GalN-hepatitis reduced significantly the velocity of the RBC phagocytosis, but not the capacity of the Mononuclear Phagocytosing System (MPS) of the liver.

3. The ANIT cholestasis reduced significantly the capacity of the MPS for RBC phagocytosis.

4. As in the undamaged perfusion experiments in both GalN hepatitis and ANIT cholestasis damaged livers phagocytosis of young RBC was significantly higher then phagocytosis of the old RBC.

598 GLUCOSE FLUX THROUGH THE HEXOSE-MONO-PHOSPHATE SHUNT DURING IN VITRO AGEING OF HUMAN SKIN FIBROBLASTS. J.F. Jongkind, A. Verkerk, M. Poot. Department of cellbiology and genetics, Erasmus University, Rotterdam, The Netherlands.

In human skin fibroblasts the glucose flux through the hexose monophosphate shunt (HMS) was calculated from the rate of $^{14}CO_2$ and (^3H) - H2O formation in the culture medium with respectively (^{14}C) - glucose labeled at the 1- or 6- position and (5-^3H) glucose as substrates. The glucose metabolism decreased from 319 to 161 n moles glucose mg protein $^{-1}$.h.$^{-1}$ upon in vitro ageing of the fibroblasts. The glucose metabolism through the HMS was 2 % of the total glucose utilization. Upon in vitro ageing of the cells, the glucose utilization through the HMS decreased to 1 % of the total glucose utilization. This decrease was not caused by a limiting enzymatic capacity, since glucose utilization through the HMS could be raised 30 fold in both "young" and "aged" fibroblasts upon stimulation of the cells with phenazine methosulphate. There was no difference in glucose utilization between proliferating and growth inhibited (confluently cultured) fibroblasts, so the effects of in vitro ageing upon glucose metabolism are not due to the differences in proliferation rate between "young" (phase II) and "aged" (phase III) human fibroblasts.

599 QUANTITATIVE MORPHOMETRY OF ZINC-IODIDE OSMIUM (ZIO)-POSITIVE SYNAPTIC VESICLES DURING AGING.

C. Giuli, C. Bertoni-Freddari, D. Paci, C. Pieri. Center of Citology, Gerontological Research Department I.N.R.C.A., Via Birarelli 8, 60100 Ancona Italy.

A morphometric investigation has been carried out on the synaptic vesicles in the cerebellar cortex of young (3 months) adult (12 months) and old (28 months) rats. The vesicles were stained by means of the zinc-iodide-osmium (ZIO) preferential staining which allows to evidence those structures filled with neurotrasmitter substances. The following parameters were measured: the number of vesicles per area (Nav), the numerical (Nv), surface (Sv) and volume (Vv) density of the vesicles, the average surface (Smv) and average volume (Vmv) of the single vescicle. An age-dependent decrease was found for Vv, Nv, Nav and Sv, whereas a significant increase was demonstrated for Vmv and Smv in the 28 months old rats when compared to the young group. Adult animals vesicles, in a comparison with those from young rats, showed significant decreases with regard to the Nav and Nv, whereas the Smv was increased.

Since ZIO-positive vesicles contain great amount of neurotrasmitters, Ca^{++} ions, Sh group, ATP and binding proteins, it has been proposed that these structures represent the "mature"-for-release synaptic vesicles. Therefore these findings, by measuring detailed morphometric parameters of the ZIO positive profiles, may help to clarify some morphological aspects involved in the age related impairements of synaptic functioning.

600 AGE RELATED NEURONAL AND GLIAL CHANGES IN THE INFERIOR OLIVE.

Luigi Gaetti (1), Filomeno Tedeschi (1), Gianriccardo Trabattoni (2), Alfredo Lechi (2). (1) Department of Pathology and (2) Neurology. University of Parma. Via Gramsci,14 - 43100 Parma, Italy.

Reduction of the brain weight up to the 6,6-15% with aging has been demonstrated by post-mortem investigations (1,2,3); more recent studies and CT scanning do generally support such findings in the elderly (4) and the view that the decrease of grey matter is directly related to the age of the subject. No recent studies are available on the senile changes in the inferior olive (I.O.). We looked for all changes likely to be set in relationship with the aging by means of histochemical methods on serial sections in a group of 32 patients (n° 10 < 60 yrs; n° 22 > 60 yrs). More particularly we studied the cytological aspects of the olivary neurons, glial cells and arteriolo-capillary district of the I.O. in the main and accessory nuclei. In the patients older than 60 years neuronal loss and isomorphous fibrillary gliosis were established. Moreover, there were frequent findings of lipofuscin intraneuronal storage and of "pigmentary atrophy". Several hypoxia patterns were present, like chromatolysis, swelling, shrinkage, Spielmeyer's homogenization. At times, aspects reminding of granulo-vacuolar degeneration were observed. The cytological aspects described seem to well justify the neuronal loss and are considered as autophagic endocellular processes, to be ascribed to local dysmetabolic trouble, in its turn connected with arteriolo-capillary derangement, even in the lack of either systemic or local angiopaty.

1) Appel FW et al. Hum.Biol.14:48,1942; 2) Dekaban AS et al.Ann.Neurol. 4:345,1978; 3) Miller AKH et al.Ann.Hum.Biol.4:253,1977; 4)Schwartz M. et al.Ann.Neurol.17:146,1985.

601 THYMIC REGULATION OF BETA-ADRENOCEPTORS DURING AGING

P. Fattoretti, A. Basso, C. Viticchi and L. Piantanelli. Centre of Biochemistry, Gerontol. Res. Dept. - I.N.R.C.A., Ancona, Italy.

It has previously been shown that thymus is capable of modulating beta-adrenergic system functions. In particular, a neonatal thymic graft exerts a corrective action on some impairments observed in old ages. Such age-related impairments are linked to a decrease in receptor density, as demonstrated in experiments with submandibular glands and brain cortex of mice of different ages. Thymic influence is also exerted via beta-adrenoceptor regulation, as a neonatal thymus grafted into old recipients is capable of correcting the alteration observed in old untreated mice. An intriguing interaction was also observed between thyroid and thymus in regulating beta-adrenoceptors. Young athymic nude mice showed an altered receptor states, altered receptor regulation following thyroxine injection. Such impairments were corrected by grafting them with neonatal thymus. Thymus, therefore, seems to play a key role in regulatory processes occuring during development and ageing.

602 THYROID REGULATION OF BETA 1 AND BETA 2 ADRENOCEPTORS DURING AGEING

C. Viticchi, G. Cognini and L. Piantanelli. Centre of Biochemistry, Gerontol. Res. Dept. I.N.R.C.A., Ancona, Italy.

In the present paper, data are reported dealing with regulation of beta-adrenoceptors induced by thyroid manipulation. The characterization of receptors was performed by using Dihydroalprenolol as labelled ligand and cold practolol as competitor specific for beta-1 binding sites.Both animal and human tissues were studied. Results showed that the majority of age-related changes in adrenoceptor density have to be ascribed to beta-1 type. It has also been demonstrated that thyroid hormones differentially regulate brain cortex beta-adrenoceptors of young and old mice. Data from human lymphocytes evidenced a fall in beta-adrenoceptor density a few days after thyroidectomy. Changes in receptor density are paralleled by serum levels of triiodothyronine. Furthermore, it has been shown that the extent of the fall increases with advancing age. Present findings, taken together, suggest that age-related changes in receptor regulation may be a key to understanding the failure mechanism of the adaptive capacity in the ageing organism.

603 FINE STRUCTURAL CHANGES IN AGING RAT THYROID.

Daniela Grandi - Institute of Human Anatomy - University of Parma - Italy

There are several studies in different species of mammals about age changes in thyroid follicle size, in the fine structure of the epithelial cell and in connective tissue. It is known that in the follicle epithelial cells, lysosomes are important for the production of Thyroxine and Triiodothyronine by degradation of reabsorbed Thyroglobulin. The present investigation concerns the age related ultrastructural changes in the follicle epithelial cell of thyroid rat with reference to lysosomes and exocytotic and endocytotic structures. Male S.D. rats ranging in age from 1-20 months were used; T_3, T_4 and TSH in the serum were radioimmunoassaied. Acid phosphatase activity was determined ultrastructurally on thyroid specimen of different age. The ultrastructural study was performed on sections from follicles of different mean diameter with histofunctional index evaluated according to Kalisnik (J. of Microscopy 95, 345, 1972). The age related changes, manifest after 12 month, primarily affect the lysosomes. Thyroid lysosomes from aged rats show alterations in number, size and morphology. These are numerous and chiefly secondary lysosomes. Moreover, large heterogeneous lysosomes containing filamentous structures, granules, membranes and lipid-like bodies are observed. Sometimes these autolysosomes and residual bodies are associated with myelin-like patterns. Other aging changes affect the follicular cell membrane with an increase of endocytotic structures (pseudopodia, colloid droplets, micropinocytotic vesicles) not always associated with exocytotic structures increase. These modifications together with the secondary lysosomes increment could explain the reduction of thyroid secretory activity in the rat.

604 **Aging of algae chloroplasts.** G.M.Voskoboinikov. Murmansk Marine Biological Institute, KFAS, USSR.

Chloroplast aging in ontogenesis of unicellular and multicellular algae possessing no differentiated tissues is displayed as a gradual decrease of the cell plastidom in volume, partial a certain cases? complete reduction of tilacoids, formation of concentric membranous structures, lessening of the number of ribosomes, accumulation of plastiglobule material, attended by diminishing ofpphotosynthetic pigment and suppression of photosynthesis. In chloroplasts of multicellular algae with differentiated tissues the period of maturity is extended. Up to the fall of the tallome parts in Sargassum, Ahnfeltia, Gracilaria no structural or functional decay in the cell photosynthetic apparatus has been hoted occur. After shedding a drastic activation of the aging process in observed. Thus, a whole complex of factors for instance, such as the morphology of organisms, characteristic mode of food supply and movement of nutrient elements, ultrastructure of photosyntheting cells is lively to be responsible for the differences ditected.

605 THE USE OF EPIDERMAL CELL CULTURES TO INVESTIGATE THE CARCINOGENIC ACTION OF BRACKEN FERN.
Ulrich E. Loening, Dept. of Zoology, University of Edinburgh, West Mains Road, Edinburgh, EH9 3JT.

Bracken (Pteridium aquilinum) is radio-mimetic and causes alimentary and bladder tumours in ruminants, in association with papilloma viruses. The viruses are being studied in several laboratories. There have been almost no studies on the mechanism of action of bracken extracts on cells, although the active agent may have been identified (Hirono et al 1984).

Hamster epidermal hair follicle cells were cultured, essentially as described by Pera & Gorman (1984). Attempts to demonstrate transforming activity of extracts of bracken will be described. This system may provide a suitable rapid assay for environmental carcinogens.

Hirono, I. et al (1984) The Vet. Record, October 1984 pp. 375-378.
Pera, M.F. & Gorman P.A. (1984) Carcinogenesis 5, 671-682.

606 **PROBLEMS IN EXTRAPOLATING GENOTOXICITY DATA FROM CELLULAR SYSTEMS IN VITRO**
Georges R. Mohn, Albert A. van Zeeland, Department of Radiation Genetics and Chemical Mutagenesis, State University of Leiden. Wassenaarseweg 72, NL-2333 AL Leiden,The Netherlands,

In recent years, the increased testing of environmental compounds for genotoxic effects, using microorganisms and cultured mammalian cells as indicators, has let to an accumulation of in vitro data on the potential mutagenic and/or carcinogenic properties of numerous compounds.

In view of the concomitantly increasing lag of time necessary for obtaining confirmation (or not) in long-term animal assays, there is a need for alternative, short-term methodologies enabling a rapid estimation of the genotoxic potential of chemical in animals.

Our approach to this subject included the following main objectives: (i) to assess the usefulness of in vitro assays for estimating the intrinsic genotoxic potency of chemicals, and (ii) to determine whether the relative genotoxic potency observed in vitro is representative of the in vivo situation. Since the answer to this last question was negative, efforts were also made to further develop the "DNA repair host-mediated assay", as a sensitive short-term test for monitoring the presence and persistence of genotoxic factors in various organs of animals.

The experiments involved quantitative comparative mutagenesis determinations using ethylating agents(*) differing in DNA reactivity, mammalian metabolism, and mutagenic potency. Under concomitant measurements of DNA dose and DNA adduct formation, the mutagenic activities of these chemicals were compared in various genes of E. coli K12 and V79 Chinese hamster cells.

The results can be summarized as follows:

1) Under standardized in vitro treatment conditions and at identical DNA dose levels, the frequencies of induced mutations observed in the present bacterial systems (forward mutations in the gyrA gene, among others) are not representative and usually lower than those induced in cultured mammalian cells (hprt gene).

2) Under the same in vitro treatment conditions, the relative mutagenic potency order is identical in bacteria and the mammalian cells, namely, ENNG > ENU > DES > EMS; DEN being not mutagenic in the two organisms without addition of mammalian (rodent) liver preparations containing Cyt-P450-dependent mixed function oxidases.

3) The extremely high mutagenic activity of ENNG in bacteria and mammalian cells in vitro is very probably due to its intracellular, glutathione-mediated activation to highly reactive chemical species.

4) Both in bacteria and the mammalian cells the frequencies of induced mutations are directly proportional to the levels of O6-ethylguanine formed in DNA after exposure to ENNG, ENU, DES, and EMS, in contrast to results obtained when other DNA adducts are used as dose parameters.

Although this does not prove (but strongly indicates) that O6-ethylguanine is responsible for the induction of premutations after treatment with ethylating agents, the present results demonstrate that this latter DNA adduct can serve as an empirical dose monitor both in E. coli and in mammalian cells.

5) The addition of mouse liver S-9 preparations (post-mitochondrial fractions) in the in vitro assays allows to determine which chemicals are likely to be substantially activated (DEN) or deactivated (ENNG) in vivo; However, the mutagenic potency order remained the same under these conditions, namely, ENNG > ENU > DES > EMS > DEN.

6) When the indicator bacteria are exposed directly to the in vivo metabolism of living animals (a methodology called "host-mediated assay"), the relative order of mutagenic potency on the basis of exposure levels becomes drastically different from that observed in vitro, using either E. coli or V79 mammalian cells, namely, DEN > ENU > ENNG > EMS.

This potency order observed when the indicator bacteria are present in the livers of treated animals is in concordance with liver carcinogenesis and DNA binding data obtained in rodents treated with the same chemicals.

These latter results indicate that ranking orders of genotoxic potency determined in vitro, regardless whether they were obtained with bacteria or mammalian cells, are not representative of the in vivo situation. Obviously, the differences observed are due to differences in the pharmacokinetic properties of the ethylating agents used in the present study, including distribution, bioactivation, and deactivation, and which lead to different doses in the tissues of exposed animals.

On the other hand, the comparative mutagenic potency results described earlier using bacteria and mammalian cells in vitro, and based on specific DNA adduct formation, strongly indicate the possibility of adequately determining the intrinsic genotoxic potency of (ethylating) chemicals in vitro.

In conclusion, it may appear feasible to use a combined short-term in vitro and in vivo approach to rapidly obtain information about (i) intrinsic genotoxic potency and (ii) distribution of genotoxic factors in organs of animals treated with chemicals, for which long-term mutagenesis and carcinogenesis data are not (yet) available.

In attempts at developing short-term, multi-organ genotoxicity assays in vivo, use was made of the "DNA repair host-mediated assay". A pair of E. coli K12 strains with various genetic markers was constructed which differ vastly in DNA repair ability (uvr+/rec+ vs. uvrB/recA), and which can be inocculated and recovered from several organs of chemically treated rodents, such as, liver, spleen, lungs, kidneys, testis, stomach, small intestine, colon, and the blood stream. Determination of induced genotoxic effects occurs on growth medium on which colonies of both strains can be identified by color morphology (white vs. red). Up to now, the assay has been calibrated with chemicals of known mutagenic and carcinogenic properties; its general usefulness as a rapid, organ-specific genotoxic dose monitor in vivo remains to be established.

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(*)Abbreviations: DEN, diethylnitrosamine; DES, diethylsulfate; EMS, ethylmethanesulfonate; ENNG, N-ethyl-N'-nitroso-N-nitroguanidine; ENU, ethylnitrosourea.

607 CYTOTOXIC EFFECTS OF TRIALKYLLEAD COMPOUNDS.

Heinz Faulstich (1), Peter Endres (1), Christos Stournaras (2), Gerd Weber (3).

(1) Department of Physiology, Max-Planck-Institute for Medical Research, Jahnstr. 29, D-6900 Heidelberg, F.R.G., (2) present address: School of Medicine, University of Crete, GR-71409 Hiraklio, Greece. (3) Max-Planck-Institute for Cell Biology, D-6802 Ladenburg, F.R.G.

Trialkyllead is a degradation product of tetraalkyllead, which is added to gasoline as an antiknocking agent. Recently, trialkyllead was shown to occur in rain, sometimes in concentrations known to be toxic for mammalian and plant cells. Several cellular structures and enzymes were found to be inhibited or destructed by trialkyllead. One of them is tubulin. For example the assembly of porc brain tubulin into microtubules is inhibited by about 50% in the presence of 5 µM trialkyllead chloride. At a similar concentration preformed microtubules were found to be destructed. It was detected that 2 out of the 18 thiol groups present in the (α+β)tubulin dimer, which are essential for the assembly reaction, are specifically blocked by triethyllead chloride.

Another system susceptible to the toxin is the Na^+/K^+-dependent ATPase located in the plasma membranes of e.g. soybean cells. It was found that 50% inhibition of this enzyme was reached at a concentration of 10 µM triethyllead chloride. The outstanding cytotoxicity of trialkyllead compounds was further demonstrated by studying the growth of cell cultures. For example, mammalian, as well as plant cell cultures stopped growing in the presence of 1 µM triethyllead chloride. Protoplasts of soybean [Glycine max.] were inhibited at a concentration as low as 0.1 µM.

Dose-response curves of the various cytotoxic activities have been established. Therefore, in principle, all of these biological systems may be used for assaying trialkyllead in environment. Specificity of the toxic action is warranted by parallel samples being monitored for their lead content, for example, by atomic absorption. Actually used for the determination of trialkyllead in rain water samples was the inhibition of microtubule assembly. This bioassay will be discussed im some detail.

608 ARE LYMPHOCYTES TRIGGERED BY AIR IONS ? - J.M. Witkowski/1/,W.Narożny/2/, J.Myśliwska/1/ and J.Jaśkowski/3/. /1/ Department of Histology, /2/ Oto-rhino-laryngol. Clinic and /3/ Department of Biophysics, Medical School, PL-80-210 Gdańsk,Poland.

Elevated concentration of negatively charged airborne particles /air ions/ present in some natural environments has long been considered as beneficial for human health, especially in certain diseases of respiratory tract. Our recent works had shown that exposition of human or animal to artificially generated "cloud" of negative air ions at known charge concentration helps wound and burn healing. However it also accelerates the speed of growth of certain experimental tumors. On the other hand, we have proved the opposite efect of positive air ions in these cases. The tonsils, part ofimmune system guarding the entrance of both respiratory and digestive tracts, remain in direct contact with the breathed air. Therefore, their function could be affected by increased concentration of air ions observed in industrial environment as well as in common household. In order to check this possibility we have exposed human tonsillar lymphocytes to either positive or negative ion clouds. Action of air ions on the cells has been directly confirmed by microfluorimetric measurement of cell surface charge /csc/ and transmembrane potential /tmp/ of cells stained with appropriate fluorescent probes /a cyanine for tmp and fluoresceinated poly-L-ornithine for scc determination/. The cell charge changed accordingly to the sign of ions applied, while observed changes in the tmp were more complex. As functional tests, the estimation of ability of ion - treated lymphocytes to form "active" and "late" rosettes with sheep red cells as well as their ability to uptake 3H-thymidine after PHA stimulation were investigated. Exposition of cells to air ions of both signs had a profound effect on "active" rosette formation, which may suggest an influence of elevated concentration of air ions on /tonsillar/ lymphocyte - antigen contact in vivo.

609 ANIMAL CELLS AS MONITOR FOR ENVIRONMENTAL FACTORS: NUTRITION, HEAVY METALS, TEMPERATURE

Volker Storch, Zoologisches Institut, D-6900 Heidelberg (West Germany)

Various cell types (e.g. hepatocytes, midgut gland cells) change their ultrastructure, and thus, their function, correspondingly to changes in the environment of the organism, e.g. food availability, accumulation of toxic substances, and temperature. Therefore, they can be used for monitoring the quality of the animal's environment (Storch, Umschau 1, 21, 1985). This technique has already been applied to some economically important fishes and shrimps, whose nutritional requirements are not known (for lit.: Vogt et al., Aquaculture 48, 1, 1985). Likewise, the method was found to work well with small isopods and amphipods, whose nutrition is only partially understood (Storch,Symp Zool Soc London 53, 167, 1984). In this regard, feeds can rapidly and reliably be tested for their suitability as food for the species as well as for the different stages of the animal's life cycle. When *Chanos* fry, for instance, were fed a variety of unicellular algae, the results showed a clear succession. Different patterns of hepatocyte ultrastructure can be demonstrated even in the third position of the food chain (Segner et al., Aquaculture 42, 109, 1984). This, however, is true on condition that these cells have not reached a stage beyond the "point of no return" (Storch and Anger, Helgol Meeresunters 36, 67, 1983). - Comparatively, other cells of the same organ (hepatopancreas) are important sites of heavy metal accumulation (Prosi et al., Zoomorphology 102, 53, 1983). This was demonstrated through X-ray microanalysis, histochemistry, and atomic absorption spectroscopy. The nutritional status of the animal strongly influences the uptake of, and correspondingly, the effect of heavy metals (Segner and Storch, Z angew Ichthyol 1, 39, 1985). - Another factor which influences the ultrastructure of fish hepatocytes is water temperature. Ultrastructural modifications have recently been complemented with biochemical data (Braunbeck, Thesis Fac.Biol. Heidelberg, 1985).

610 ALUMINUM LOCALIZATIONS IN AND EARLY DIGESTION OF SOYBEAN BACTEROIDS. L. Evans Roth, Gary Stacey, J.R. Dunlap, N.S. Dawson, University of Tennessee, Knoxville, Tennessee, 37996-0810, USA.

Aluminum, long known to be detrimental to soybean productivity, has been localized in the polyphosphate granules (PPG) of bacteroids in root nodules of soybean. Plants were grown in the laboratory without aluminum being added to the medium that was at pH 6.8 and were innoculated with strain 110 or 61A101C of Bradyrhizobium japonicum; nodules were collected on days 20 to 23, fixed in glutaraldehyde and osmium tetroxide solutions, dehydrated in acetone, and embedded in Spurr's medium. By using electron microscopy and energy-dispersive X-ray analysis, bacteroids in early infections were shown to have typical PPG constituents: phosphate, calcium, and magnesium; if bacteroids were tested in cells that had been infected longer, aluminum was also present in the PPG. Bacteroids are engulfed by and digested in degradation vacuoles, several of which are typically found in normal young cells and are always located so near the host-cell nucleus that the nuclear envelope and vacuole membrane are in close apposition in certain areas; numerous bacteroid remnants in these vacuoles include PPG in which aluminum was also detected. These results show that aluminum accumulates in PPG only after organisms have been resident in host cells and was present when bacteroids were phagocytozed.

Cells with early infections were degrading bacteroids at the same time that new infection threads were invading their cytoplasms. This unexpected finding suggests that a more rapid turnover of bacteroids is taking place than is usually expected and that bacteroid death, though it may result from nodule senesence, may also be otherwise determined.

There are two interpretations of these findings. First, aluminum may be the marker that allows the demonstration of normal bacteroid degradation and turnover. Second, aluminum toxicity may cause early bacteroid death by accumulating in PPG and DNA; aluminum is known to bind to DNA detrimentally, and the poor productivity of soybeans and other leguminous crops in soils where stress conditions include acidity and readily available aluminum would then be caused by the premature deaths of bacteroids.

611 DOSE-RELATED INDUCTION OF RAT HEPATIC DRUG METABOLIZING ENZYMES BY SUBSTITUTED UREA HERBICIDES.

Bernadette Schoket, I. Vincze. Department of Biochemistry, National Institute of Hygiene, Budapest, Gyáli ut 2-6., H-1097 Hungary

Several substituted urea herbicides are potent inducers of rat hepatic drug metabolizing enzymes as it has been found in our investigations. Among these herbicides those compounds which contain two halogen substituents on their phenyl group cause greater induction of cytochrome P-450 /P-450/ linked benzo/a/pyrene monooxygenase /BP-MOO/, 7-ethoxycoumarin O-deethylase /ECOD/ and 7-ethoxyresorufin O-deethylase /EROD/ activities than those which carry one halogen or no one. There is not such a significant relationship between the induction of epoxide hydrolase /EH/, UDP-glucuronyltransferase /UDPGT/ and glutathione S-transferase /GST/ and structural differences of the compounds. A dichlorinated /diuron/ and a monochlorinated compound /chlortoluron/ has been applied to measure dose-related induction. It has been found that "saturation" of the induction system of P-450 content, BP-MOO, ECOD and EROD activity may occur at similar molar doses, and in the case of the dichlorinated compound at much higher activities. Induction of EH, UDPGT and GST activities also describes saturation curves in the function of molar doses. Curves of the two compounds run together and the structural difference is not reflected in the enzyme inductions.

612 SUBCELLULAR LOCALIZATION OF PLUTONIUM-241 IN CHINESE HAMSTER LIVER BY ELECTRON MICROSCOPIC AUTORADIOGRAPHY.

Arnulf Seidel, Gerhart Hotz. Kernforschungszentrum Karlsruhe, Institut für Genetik und Toxikologie, Postfach 3640, D-7500 Karlsruhe 1, FRG.

Actinides are rapidly eliminated from rat liver, presumably by biliary exocytosis of lysosomes, while hamsters, dog and man retain these elements for a very long time in their liver. The aim of our studies is to elucidate the reasons for these species differences. Citrate complexes of Pu-241 were injected into Chinese hamsters. After 4,84 and 245 days electron microscopic autoradiography with liver sections was performed. With its soft beta emission Pu-241 is the only actinide isotope suitable for this method.

From the 4th day onwards the beta-tracks were consistently found over osmiophilic globular structures in hepatocytes of about 1 µm diameter. These structures were randomly distributed in the cytoplasm.There was no change with time up to the 245th day. At day four after Pu-241 injection, beta-tracks were also regularly seen over chromatin-rich regions of hepatocyte nuclei. There was no indication of any specific accumulation in endothelial lining cells or Kupffer cells with time.

We have shown previously (e.g. Seidel et al., Eur. J. Cell Biol. 37 (1985) 89) that in Chinese hamster liver Pu becomes bound within ~5 days to particles of ~1 µm diameter which physically (density, electrophoretic mobility) and enzymatically fulfil all criteria for lysosomes. We conclude from these and the present morphological results that the structures to which the Pu-241 beta-tracks are associated represent lysosomes, which obviously cannot be excreted by the Chinese hamster hepatocyte. Binding of a significant fraction of the hepatic Pu to cell nuclei is also confirmed by our biochemical results.

613 EFFECTS OF WATER-SOLUBLE OIL FRACTIONS ON THE HEART OF MARINE MOLLUSCS.

S.N.Nistratova (1), L.V.Berdysheva (1), R.Perez (2), R.Valle (2). (1) Institute of Developmental Biology, USSR Academy of Sciences, Moscow, USSR, and (2) Institute of Oceanology, Academy of Sciences of Cuba, Habana, Cuba.

The effects of water-soluble oil fractions (WSFs) have been studied on the isolated heart of gastropods Strombus gigas and Bursatella leachi, in particular their effects on the myocardium and on its interaction with neuro transmitters: acetylcholine (ACh) and serotonin (5-HT). The WSFs were shown to effective even when very diluted. The stock solution (10^{-3}) was obtaineed by dissolving 1 ml of crude oil in 1 liter of sea water. The WSFs at concentrations of 10^{-8} to 10^{-7} and 10^{-4} to 10^{-3} increased the amplitude and frequency of heart contractions but at concentrations of 10^{-6} to 10^{-5} induced the cardiac arrest. These concentrations correspond to those occurring when the oil is dumped into the sea. The Ach effect was partially or completely blocked against the background of the stimulating effect of the WSFs and the inotropic effect of 5-HT was blocked at higher concentrations of the WSFs (up to 10^{-3}). The higher is the heart sensitivity to the transmitters, the stronger is the damaging effect of the WSFs. The data obtained suggest that oil, even when very diluted, affects the activity of the cardiac muscle and its nervous regulation.

614 CHANGES OF TRIGONELLINE AND FORMALDEHYDE LEVEL IN TOMATO MUTANTS

György Bisztray, István Molnár, Ernő Tyihák. Plant Protection Institute of Hungarian Academy of Sciences, H-1525 Budapest, POB 102, Hungary

Trigonelline, an N-methylated derivative of nicotinic acid is a newly recognised plant hormone, which arrests cells in G2 phase of the cell cycle in many plant species. It is supported, that there is a correspondence between the change of the level of trigonelline and formaldehyde during the light treatment of plants. Different tomato mutants were illuminated with intensive light and formaldehyde liberated during the light treatment was binded with dimedone. The trigonelline and dimedone-binded formaldehyde content of the light treated and non-treated samples were determined by over-pressured layer chromatography. It was found, that the trigonelline level of dwarf mutants was higher than that of normal phenotypes. At the plants treated with light a consistent rise of dimedone-binded formaldehyde content was observed and the rise of formaldehyde content was especially expressive at the dwarf mutants.

615 EFFECTS OF HEAVY METAL ACCUMULATION ON THE MIDGUT GLAND IN A TERRESTRIAL ISOPOD, *PORCELLIO SCABER*.

Katalin Szlávecz /1/, László G. Kőmüves /2/, Sándor Gueth /3/. /1/Department of Systematic Zoology and Ecology, /2/ Department of General Zoology, /3/ Department of Atomic Physics, Eötvös University, Puskin u.3, H-1088 Budapest, Hungary.

The effects of lead and cadmium on the lysosomal enzyme activity,the protein content, and the ultrastructure of the midgut gland, which plays a central role in the metabolism of isopods, were studied. Hornbeam litter was sprayed with Pb acetate or Cd chloride solutions of different concentrations. The animals were fed with the contaminated leaf litter for one or two months. Leaf litter sprayed with distilled water served as control. X-ray fluorescence analysis showed considerable heavy metal accumulation in the whole body of the experimental animals. Total protein content of the midgut gland of the treated isopods was approximately 60 % less than that of the controls. However, the total activities of the lysosomal enzymes /acid phosphatase, acid β-galactosidase, acid glucosidase/ were not affected by the treatments, meaning, that specific activities of the these enzymes increased. Electron microscopic investigation of the midgut gland revealed characteristic ultrastructural changes in the different cell types. The amount of glycogen and the number of lipid droplets and secretory granules decreased, whereas an increase in the number of autophagic vacuoles and large secondary lysosomes was observed.

The study indicates, that heavy metals ingested with food are not only stored in the midgut gland, but they influence the ultrastructure and functions of its cells, as well.

616 HEAVY METAL (Hg, Cd) EFFECTS ON THE ULTRASTRUCTURE OF THE CHEMORECEPTOR ORGANS OF THE FISH Alburnus alburnus.

L. Hernádi (1), Raisa Pevzner (2), J. Salánki (1). (1) Limnological Research Institute of the Hungarian Academy of Sciences, Tihany, Hungary and (2) Sechenov Institute of Evolutionary Physiology and Biochemistry, Laboratory of Evolutionary Morphology, USSR Academy of Sciences, Leningrad, USSR

The ultrastructural alterations of the olfactory organs and taste buds of the bleak were studied with scanning electron microscope following treatment with different concentrations of Mercury and Cadmium. The metals were administered to the lake-water in the forms of $HgCl_2$ and $CdCl_2$. The concentrations (100 ug/l Hg^{++} and Cd^{++}, as well as 300 ug/l Hg^{++} and Cd^{++}) were kept constant. Samples were taken after exposures of 1 day, 2 days, 1 week, 2 weeks. The olfactory organs proved to be more sensitive than the taste buds to both metals upon any applied concentration and exposure times. The receptor cells of the olfactory organ showed different alterations to the different metals. The mercury treatment evoked dramatic decrease in the number of receptor cells and ciliated epithelial cells, while Cadmium only brought forth unusual deformation and destruction of the receptor cells, without affecting the ciliated epithelial cells. On the taste buds Mercury treatment affected the microridge system on the epithelial cells as well as the microvilliar system of the supporting and receptor cells. The effects showed duration and concentration dependency. Cadmium effect on the taste buds showed no structural alteration, only the mucous secretion increased over the surface of the taste buds.

617 ERYTHROPOIETIC CHANGES IN MAN AFTER HYPOKINESIA AND PHYSICAL EXERCISE.

Yan G. Zorbas, Evgueni M. Lekarev, Alexandru E. Sandu, Hypokinetic Physiology Laboratory, European Institute of Environmental Cybernetics, GR-162 32 Athens, Greece

It was suggested that after the exposure to hypokinesia (diminished muscular activity) and during the readaptation period (RP) erythrocyte balance may undergo substantial changes. Against this background the objective of this investigation was to examine the mechanism of erythrocyte changes at the cellular level during the RP of 40-days and after the exposure to different periods of combined hypokinesia (HK) and physical exercise (PE) on 6 healthy male volunteers aged 19-23 years. They were divided into three groups with 3-men in each. The examined men were subjected to combined HK and PE for 288, 360 and 525 days. For the simulation of the hypokinetic effect the men were kept under a rigorous bed rest regime. Some parameters of erythropoiesis as well as erythrocyte balance were determined during the 40 days of the RP and after the exposure to different periods of HK. During the RP cytokinetic and morphological changes in erythropoiesis were demonstrated. The amount of circulating erythrocytes and their life time was decreased. The shortening of the life time of erythrocytes was accompanied by increased proliferative activity of erythroid cells. The erythrocyte balance was not achieved till the end of the RP. The use of PE failed to prevent the development of alterations in the examined parameters of erythrocytes. It was concluded that substantial disturbances were demonstrated in erythrocyte balance during the RP and after the exposure to HK, while at the same time it was revealed that PE cannot be used to counteract effectively the development of adverse reactions of erythropoietic parameters in man after diminished muscular activity conditions exposure and during the readaptation period.

618 ACETYLCHOLINESTERASE ACTIVITY OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES IN DEMENTED PATIENTS.

Emilia Bartha(1), Katalin Szilágyi(2), Judit Szelényi(3), Susan Hollán(3), Angéla Gyévai(1). (1) Institute of Experimental Medicine, Hungarian Academy of Sciences, H-1450 Budapest, POB 67, (2) National Institute for Nervous and Mental Disease, H-1281 Budapest, Vörösháadsereg u. 116, and (3) National Institute of Haematology and Blood Transfusion, H-1502 Budapest, Daróczi u. 24, Hungary

Memory is property of both the immune and central nervous system, and progressively impaired in senile dementia of Alzheimer's type (SDAT). Acetylcholine, one of the neurotransmitters is thought to have primarily role in higher mental activity. To test the hypothesis that this disease of brain stems from or is reflected in blood cell membranes we have started to examine acetylcholinesterase (AchE) activity of red blood cells and lymphocytes in different type of demented subjects.

The AchE activity of lymphocytes in senile dementia of Alzheimer's type and mixed type were significantly lower compared to the age-matched healthy volunteers, but less variation was observed in vascular type, multi-infarct dementia (MID). Interstingly, MID patients receiving antipsychotics had higher enzyme activity. Reduction of dopaminerg inhibition by antipsychotic drugs may explain the higher AchE activity. While the difference of AchE activity of lymphocytes among subgroups proved to be statistically significant, changes in red blood cell AchE activity could not be noted.

The conclusion can be drown from our data, that in dementia there are relationship among clinical, mental status and AchE activity changes on lymphocytes, and neuroleptics have effect and are reflected on its cholinergic system.

619 PRENATAL ALCOHOL EXPOSURE AND ULTRASTRUCTURE OF CEREBRAL CORTEX NEURONS IN OFFSPRING

Elida N. Popova. Brain Research Institute of the All-Union Mental Health Center of the USSR Med. Academy, per. Obukha 5, Moscow 107120, USSR.

Clinical data demonstrate high degree of risk of fetal alcohol syndrome following prenatal alcohol intoxication. But the structural basis of brain activity disorders in offspring following prenatal alcohol intoxication needs further investigation. Electronmicroscopic study of sensomotor cortex of 21-, 30-, 60-days old rats, born of animals intoxicated during pregnancy (2 g/kg alcohol on body weight) show signs of impaired maturation of some cortical neurons. The distrophic changes have been found: swelling and oedema of single cisterns of granular endoplasmic reticulum and of Golgi apparatus, sometimes with destruction of limited membranes, appearance of lysosomes and lipofuscin. Many free ribosomes can be seen in cytoplasm. There are swelling of dendroplasma, number decrease of microtubules, desorganization, vacuolization, reduction of mitochondric cristae, vacuolar, membranous and mielinlike structures. Electron dense dendrites are also present. These changes are more distinct in 21-day-old experimental animals. Later the signs of reparative processes become more significant, but despite their presence ultrastructural changes of cortical neurons are observed up to the period of sex maturation. It is suggested that the brain damage in offspring is associated with the pathology of dendrites.

620 THE CONDITION OF INTERCELLULAR INTERACTION IN AUTOIMMUNE INSULITIS.
Brindack O.I., Poltorack V.V., Ladogubets E.V. Kharkov Endocrinology and Hormone Chemistry Research Institute, Kharkov, USSR

Intercellular interactions in pancreas of Chinoilla rabbits suffering from autoimmune insulitis induced by the administration of antiinsulin guinea pigs serum for three days have been studied. Pancreas have been fixed in Carnua and Buen liquids. Histologic preparations made of paraffinic blocks were stained with hematoxylin and eosin and for the identification of B- and α -cells of insular apparatus Homory method in Bell modification have been used. Immunohistochemically in the insular apparatus cells insulin, somatostatin and glucagon have been also revealed. Quantitative evaluation of the synthetic activity in insular cells has been done by the method of microspectral luminescent analysis. It has been found that in B-cells of experimental animals dystrophic changes and their lowed in comparison with control synthetic activity have been observed. At the same time α -cells glucagon production and δ -cells somatostatin one have been enhanced. In those islets, where parallel with dystrophic changes in B-cells, there have been the phenomena of lymphocellular infiltration, somatostatin synthesis has been higher and glucagon one rather lower than that in the islets without infiltration.

The subject concerning the condition of intercellular interactions in pancreatic islets with the autoimmune insulitis and a possible somatostatin role in the development of autoimmune component with the pathology has been discussed.

621 AUTOANTIBODIES: TOOLS IN CELL RESEARCH – CELL RESEARCH: A TOOL FOR STUDYING AUTOIMMUNE DISEASES
J. Schlammadinger (1), L. Czirják (2), Gabriella I.-Bognár (1), Edit Bodolay (2), Gy. Szegedi (2). (1) Institute of Biology and (2) 3rd Department of Medicine, University Medical School, H-4012 Debrecen, Hungary

A systemic survey of sera obtained from patients suffering from autoimmune diseases has indicated that not only the detectability of "traditional" antinuclear autoantibodies (ANA), but also the presence of anticytoplasmic activities are not too uncommon features of these illnesses. Studying sera predominantly of progressive systemic sclerosis (PSS) and mixed connective tissue disease (MCTD), anticytoskeletal autoantibodies of different specificities were demonstrated by conventional indirect immunofluorescence technique. Consequently we attach importance to the collection and characterization of relevant sera. They may serve as additional reagents in studying the elements and organization of the cytoskeleton besides the well proven battery of the already existing monoclonal antibodies. From the clinical point of view, our observations underline the need for a rather wide range of cell substrates (monolayers and other specimens) to detect all the possible autoantibody specificities, as it has already been shown that the expression of cytoskeletal, first of all intermediate filament proteins, is cell type- and differentiation-dependent phenomenon.

622 CYTOLOGICAL AND HISTOENZYMATICAL EVIDENCES OF THE PROTECTIVE EFFECTS OF ASCORBIC ACID IN DIABETIC RATS.
Victoria M.Rusu (1), I.B.Molnár (2),Nina Sildan (1), J.Madar (1), Ana Ilonca (1)and Beatrix Pal (3). (1) Biological Research Center,3400 Cluj-Napoca,Romania (2) Department of Psychiatry Pest megyei Tanács V.B.Kórháza, 2143 Kerepestarcsa, Hungary, (3) Chemical Pharmaceutical Research Institute, 75669 Bucharest, Romania.

Diabetes was induced 10 days prior to experiments by injecting i.v.a single dose of 6.5 mg/100 g b.w. streptozotocin in normal and ascorbic acid treated albino rats. Ascorbic acid was given i.p. in daily doses of 500 mg/kg b.w.,5 days before and 10 days after streptozotocin administration. Thoracic aorta, kidney and liver pieces were quickly removed and prepared according to usual techniques for cytological and histoenzymatical examination.

As compared to the control group, diabetes induces, besides typical morphological changes, deep alterations in the histoenzymatical aspects in all examined tissues. Most relevant are the increase of Glucose-6-phosphatase and Glucose-6-phosphate dehydrogenase activities in liver and the fall of succinate dehydrogenase,Cytochrome oxidase, Adenosine triphosphatase, Adenylate Cyclase,Glucose-6-phosphatase activities in kidney. At the level of aorta wall it is worth mentioning the decrease in lactate dehydrogenase and lipase, concomitently with an increase in Steroid dehydrogenase and 5-nucleotidase reaction. In ascorbic acid treated rats most of these changes induced by diabetes are significantly reduced. The findings are discussed together with glycemia, tissue glycogen content and glicosuria changes and they suggest that the beneficial effect of ascorbic acid is due to its interference with the cellular energetic processes.

623 HISTOCHEMICAL AND ELECTRONMICROSCOPICAL OBSERVATIONS ON THE SPLEEN OF DIABETIC LEWIS RATS AFTER SYNGENIC ISLET TRANSPLANTATION.

F. WOHLRAB (1), BARBARA WILKE (2), S. SCHMIDT (2), L. COSSEL (1). (1) Institute of Pathological Anatomy, Karl-Marx-University, GDR 7010 Leipzig, Liebigstr. 26, and (2) Central Institute for Diabetes "Gerhardt Katsch", GDR 2201 Karlsburg.

For islet transplantation experiments in the syngeneic system of Lewis rats adult female LEW 1 W/MaxK rats were made diabetic by a single injection of streptozotocin (50 mg/kg body weight). Pancreatic islets were prepared from neonatal (8 to 12 days) LEW 1 W/MaxK rats using the collagenase method and cultured in TCM 199 (10 mmol/l glucose, 10% NCS) at 37 °C in an atmosphere of 95% air and 5% CO_2 for 4 days. About 800-1000 islets were transplanted by injecting the islets into the splenic tissue. After transplantation plasma glucose was measured dayly untill the third week and thereafter weekly up to 200 days. The plasma glucose levels of the diabetic recipients decreased within 1 to 8 days and converted into permanent normoglycemia. 200 days after transplantation the spleen was removed and investigated by histochemical and electronmicroscopical methods. The implanted islets are morphologically and functionally intact. No inflammatory infiltrates around the transplanted islets are observed. In B cells of islets insulin was demonstrated histochemically (Victoria Blue B; pseudoisocyanine chloride) and immunohistochemically. Individual cells, however, are also reactive in orthologic structures of the spleen after preoxidation and staining by Victoria Blue B, but not by immunohistochemical insulin demonstration. It was concluded that in the cells of activated spleen also substances containing SS-groups are reactive with the dye. There come into consideration immunoglobulines (possibly the conductor protein of the hormone splenin ?).

624 THE ROLE OF LIVER SINUSOIDAL ENDOTHELIAL CELLS IN THE TRANSPORT OF CELLULAR DEBRIS IN CHOLESTASIS. Liliane Dubuisson(1), Paulette Bioulac-Sage(1), André Quinton(2), Liliane Boussarie(1), Charles Balabaud(1,2). (1) Lab. Inter. Cel. Univ. Bordeaux II. (2) Service Mal. Ap. Dig., Hôpital St André, 33000-Bordeaux FRANCE.

Cellular debris, consequence of hepatocyte death, are phagocytized by macrophages. Debris are often seen in the Disse space and in the sinusoidal lumen. The aim of this study was to understand how cellular debris reached the lumen.

7 patients with cholestasis (from severe to mild) of various origin were studied. The level of aminotransferases was either normal or mildly elevated. A liver biopsy was fixed for routine histology and perfusion-fixed for electron microscopy.

In areas of cholestasis, sinusoidal macrophages contained diastase-PAS positive material. In zones chosen for electron microscopy, hepatocyte plates, sinusoid architecture and the endothelial wall were well preserved. Some cellular debris were observed not only in the Disse space and the sinusoidal lumen but also in endothelial cells. The complete sequence in the transport of cellular material from the Disse space to the sinusoidal lumen seems to follow the following steps : 1. Blebs formation at the sinusoidal membrane of hepatocytes. 2. Engulfement of cellular debris or blebs by endothelial pseudopodes. 3. Transport by the endothelial cell. 4. Release at the luminal side. Occasionally, bile pigments were also transported through this mechanism. In all cases, at least one step of the sequence was seen. Under condition of mild hepatocellular damage without rupture of the endothelial wall, the endothelial transport might not be the only one to get rid of the debris ; indeed, occasionally, macrophages were seen in the Disse space. On the opposite, widening of endothelial pores was not observed.

The mechanism of the transport as well as its real physiological importance are yet unknown. It was also observed in areas with a ruptured endothelial wall suggesting that it truly represents a function of this cell at least in cholestasis.

625 TRANSFORMATION OF LIVER PERISINUSOIDAL CELLS INTO FIBROBLASTS IN AGNOGENIC MYELOID METAPLASIA. Paulette Bioulac-Sage(1), Dominique Roux(2), André Quinton(2), Hervé Lamouliatte(2), Charles Balabaud(1,2). (1)Lab Inter Cel Univ Bordeaux II. (2)Ser Mal Ap Dig, Hôp St André, Bordeaux FRANCE

In agnogenic myeloid metaplasia (AMM) the liver resumes an erythropoietic activity. Erythropoietic foci have been made responsible for portal hypertension (of sinusoidal origin). The mechanism remains however poorly understood. It has been proposed recently that sinusoidal fibrosis could also represent an important factor.

4 consecutive patients with AMM were studied. A liver biopsy (needle or surgical) was fixed for routine histology (hematoxylin-eosin, Sirius red) and perfusion-fixed for electron microscopy (1.5% glutaraldehyde).

The following observations were made. Under light microscopy : 1. moderate sinusoidal dilatation. 2. Sinusoidal infiltration by megakaryocytes (3/4). 3. Perisinusoidal fibrosis on the Sirius red staining (4/4). Under electron microscopy : 1. Presence of myeloid cells in sinusoids and Disse space (4/4) with mainly erythroblasts and megakaryocytes. 2. Widening of the Disse space with numerous and thick bundles of collagen. 3. Transformation of perisinusoidal cells into fibroblasts containing less lipids, active RER, increased number of microfilaments sometimes condensed below the plasma membrane, long and thick processes. 4. Occasional fragments of basement membrane-like material were seen below the endothelial wall.

From these results it is proposed that other factors than infiltration of sinusoids by myeloid cells can play a role in portal hypertension : collagenisation of the Disse space and transformation of perisinusoidal cells into fibroblasts are additonal factors susceptible to increase sinusoidal resistance. Based on the development of myelofibrosis, it is assumes that liberation of growth factor by megakaryocytes components stimulates perisinusoidal cells proliferation and transformation into fibroblasts and increases their collagen secretion.

626 RELATIONSHIP BETWEEN CALMODULIN ACCUMULATION AND OTHER PRE-REPLICATIVE EVENTS DURING LIVER REGENERATION. Mª.J.Pujol, Mª.R. Piñol, Mª.J.Coll, M. Soriano and O. Bachs. Dpt. Histology and Cell Biology. Fac. Medicine. University Barcelona. Spain.

Several lines of evidence indicate that a great variety of cellular activation processes are mediated by Ca^{2+}-Calmodulin complex formation. A cytosolic surge of Calmodulin (CaM) is observed between 8-12 h. after partial hepatectomy.A surge of cytosolic cyclic AMP between 12-14h. and a surge of cyclic AMP dependent protein kinase activity between 14-16 h. have also been observed. These three surges are necessary events to initiate DNA synthesis at 18 h. after surgery.

To find out if cyclic AMP and cyclic AMP dependent protein kinase surges are dependent of the previous surge of CaM, the effect of the Ca^{2+} - Calmodulin bloker, Trifluoperazine (TFP),on these processes has been studied.

The results show that TFP produce a delay of 12 h. in cytosolic CaM surge, a 50% inhibition on cyclic AMP intracellular accumulation and a total inhibition in the activities of cyclic AMP dependent and independent protein kinases.

On the other hand, it has also been determined whether effect of TFP is specific Ca^{2+}- Calmodulin complex or if TFP acts directly on protein kinase activity. The study has been carried out adding different concentrations of this drug to the incubation medium for protein kinase determination. Results show that TFP inhibits in a dose dependent way the cyclic AMP dependent protein kinase activity.

The results obtained indicate that CaM is partially responsible for the intracellular cyclic AMP accumulation observed at 14-16 h. after partial hepatectomy. Since TFP inhibits directly cyclic AMP dependent protein kinase activity nothing can be concluded about the relationship between CaM accumulation and cyclic AMP dependent protein kinase activity surge using this drug.

627 CHANGES IN THE CYTOCHEMICAL ACTIVITY OF SEVERAL PHOSPHATASES IN HEPATOCYTES FROM RATS CHRONICALLY EXPOSED TO ETHANOL.
F. Mayordomo (1), C. Gómez-Perretta (1), C. Guerri (2), R. Sanchis (2). (1) Centro de Investigación, Hospital La Fe, Avda. Campanar 21, 46009 Valencia, and (2) Instituto de Investigaciones Citológicas, Amadeo de Saboya 4, 46010 Valencia, Spain.

In the present work we have analyzed the cytochemical activity of several phosphatases of lysosomes, endoplasmic reticulum, and plasma and nuclear membrane of periportal hepatocytes from alcoholic rats. Cytochemical activity of AcPase (lysosomes), AlkPase (plasma membrane of bile canaliculi) and G6Pase (nuclear membrane and ER),was detected in the hepatocytes of both control and alcoholic rats. However, the density of the deposits as well as their relative volumes were lower in the ethanol than in the control groups for all the enzymes studied, suggesting that chronic ethanol ingestion modifies the function of lysosomes, plasma and nuclear membranes and ER in the hepatocytes of male adult rats.

628 EFFECTS OF ETHANOL ON THE ACTIVITY OF SEVERAL PHOSPHATASES IN HEPATOCYTES FROM FETAL RATS.
C. Gómez-Perretta , F. Mayordomo , V. Cortés , E. Blas. Centro de Investigacion, Hospital La Fe, Avda. Campanar 21, 46009 Valencia, Spain.

We have analyzed the cytochemical activity of several phosphatases of Golgi apparatus, endoplasmic reticulum and lysosomes of periportal hepatocytes from rats prenatally exposed to ethanol, using qualitative and quantitative electron microscopy and cerium ions as capture agent.

Cytochemical activity of NDPases (IDPase, UDPase), AcPase and TPPase was detected in all hepatocytes both from control and treated fetuses, but the density and relative volume of the precipitates were always lower in the latter group of animals. The deposits of the electron-dense cytochemical markers were localized in: the ER and "trans" region of the Golgi apparatus for both NDPases; lysosomes and the GERL system for the AcPase; and the "trans" Golgi cisternae and GERL for the TPPase. In all cases the stereological quantification of the cerium precipitates shows that there were significant differences ($p < 0.05$) between the alcohol exposed fetuses and the control group, suggesting that ethanol is probably causing some impairment of the function of these organelles.

629 SOME REGULARITIES IN THE RESPONSES OF MAMMALIAN INTESTINE EPITHELIOCYTES TO THE ACTION OF CHOLERA TOXIN. V.A.Shakhlamov, Institute of Human Morphology, USSR Academy of Medical Sciences, Moscow, USSR.

The effect of cholera toxin (choleragen) on intestinal epitheliocytes of rabbit-sucklings, as well as of adult rats and guinea pigs was studied 10min-24h following intraluminal injection of the toxin using transmission electron microscopy, cytochemistry and immunocytochemistry. It was shown that all the types of epitheliocytes studied (absorbing and goblet cells, Peyer's patch marginal cells and endocrine cells) bind choleragen by their plasmalemmal receptors (G_{m1}) but respond differently to its effect; these different responses of the cells were dependent on their differing functions and were characterized by appropriate ultrastructural manifestations. Cholera toxin produced focal alterations in the epithelial layer of the small intestine, while in the colon the epithelium was damaged almost totally. Absorbing cells of the small intestine localized in the damaged areas secreted large amounts of fluid into the lumen, while in the colon the epitheliocytes greatly reduced their absorbing capacity. In the goblet cells of both the small intestine and the colon, enhanced mucus secretion was observed. Peyer's patch marginal cells transported the antigen (choleragen labelled by horse radish peroxidase) to the zone of localization of lymphocytes and macrophages. Among the endocrine cells, the most succeptible to choleragen action were enterochromaffine cells (Ec-cells) of the small intestine which almost immediately (within 10min) responded by massive exocytosis of serotonin-containing granules into the areas of locally damaged epithelium exerting a paracrine effect on brush-border cells.

630 IMMUNOLOGIC CHARACTERISTIC OF PALATINE TONSILS IN CHRONIC TONSILLITIS. A.S.Aznaurian, A.K.Shukurian. Erevan Medical Institute, 375025 Erevan, Kirov str 2, USSR.

In patients of different age with chronic tonsillitis 100 palatine tonsils were studied. Lymphoid tissue immune reactivity and the dynamics of stromal morphological changes were estimated. Different immunohistochemical methods were used for investigation of the material.

The results got indicate that the intensity of the antibody forming process closely correlates with age and duration of the disease. Immunomorphologic analysis of lymphoid cells confirms the prevailing content in the tonsils of B-lymphocytes responsible for humoral immunity. It is also confirmed by high alkaline phosphatase activity. Certain regularity of bound immunoglobulin distribution was revealed according to luminiscence intensity of tonsillic tissues depending on the form of chronic tonsillitis. In addition to that, there is an abrupt change of the microcirculatory bed histostructure in different age groups due to sclerosis and obliteration.

631 IMMUNOPATHOLOGICAL STUDY ON THE SKIN OF PATIENTS WITH MIXED CONNECTIVE TISSUE DISEASE.

Wang Wanchao, et al. Dept. of Immunology, Shanghai No.2 Medical University
Dept. No.1 of Basic Immunology, Shanghai Institute of Immunology

The results of immunopathological study of mixed connective tissue disease (MCTD, 14 cases) and other connective tissue diseases (CTD, 24 cases) are reported in this paper.

All the 14 cases possessed the clinical diagnostic essentials of MCTD suggested by Sharp, and all were fluorescence staining (+) of epithelial cell nuclei in the skin. Therefore, we think that MCTD is an autoimmune disease, and is also a separate disease which is different from other CTD.

Three kinds of fluorescent staining method on epiderma cell nuclei were compared. i. e. 1. paraffin digestion 2. paraffin section 3. consistent low temperature cryo-section (-20° C). The results demonstrated that the cryo-section method was the best. When combined with clinical and other immunological characteristics, it can be used as one kind of reliable indicators for diagnosing MCTD. Finally, the kinds and mechanisms of immunoglobulin deposits on epiderma cell nuclei are also discussed.

632 EPITHELIAL CELL INJURY OF HUMAN CONJUNCTIVA IN THE CASE OF KERATO-CONJUNCTIVITIS SICCA COMBINED WITH POLYARTRITIS CHRONICA PROGRESSIVA

J. Serfőző (1), Magdolna Török (2), Ágnes Tóth-Jakab (1). (1) Institute of Biology, L. Kossuth University, H-4010 Debrecen, and (2) Department of Ophthalmology, University School of Medicine, H-4012 Debrecen, Hungary

During the development of "dry eye syndrome", similarly to another inflammatory diseases, ensue a lot of structural alterations in the epithelium of human conjunctiva. These, however, were not characteristic of diseases, in question. In the case of keratoconjunctivitis sicca combined with polyarthritis chronica progressiva, on the other hand, there appear two remakable phenomena which occur rarely or not at all in the early and later stages of "dry eye syndrome". Namely, the intensity of secretory vesicle's formation decreased in the superficial epithelial cells, and the formation of myelinoid bodies increased, mainly in the strongly keratinized (dark) epithelial cells. The latter symptom was a characteristic feature of this desease.

The myelinoid bodies have thread-reel structure forming, generally, in the apical cell regions, seldom near the nucleus. These are sorrounded by a large and empty space in the cytoplasm bordered by a membrane. One or more extensions protrude from the membrane, and then they become thin and wind terminally assuming a characteristic thread-reel structure. Generally, these dense bodies exist as twin figures. When keratine content of light epithelilal cells increases, in the course of light-dark cell's transformation, the dense figures leave the cells by means of exocytosis. These dense figures are scattered in the dilated extracellular spaces where they phagocytised and accumulated by invasive macrophages.

633 **THE LOCALIZATION OF ACID PHOSPHATASES IN THE ENDOTHELIAL CELLS OF BLOOD CAPILLARIES UNDER THE EFFECT OF CARDIOTOXIC DOSES OF CATECHOLAMINES.**

S.A.Sisakyan, Erevan Medical Institute, Erevan, USSR.

Gomori's method modificated by us makes possible the determination of acid phosphatase activity and demonstrates the presence of the enzyme in the endothelial cells of myocardial blood capillaries of experimental animals (rats, mice, guinea pigs). Following prolonged incubation of the sections the cells of capillary wall were stained diffusely. After the treatment by toxic doses of catecholamines, the contours of the endothelial cells, as well as their nuclei were clearly delineated, which was indicative of an increase in the activity of the enzyme and enhancement of the permeability of cellular membranes.

634 CALMODULIN INHIBITION PROTECTS ISCHEMIC MYOCARDIUM FROM REPERFUSION INJURY.

Ján Slezák, Ivan Gabauer, Attila Ziegelhöffer, Ján Slezák, Jr. Institute of Experimental Surgery, Center of Physiological Sciences, Slovak Academy of Sciences, 842 33 Bratislava, Czechoslovakia

Reperfusion of myocardium after ischemia exceeding 40 min results in morphological, physiological and biochemical changes induced by enhanced cytosolic Ca^{2+} concentration.

It has been suggested that altered membrane integrity caused by degradation of membrane phospholipids secondary to ischemia is the most important factor responsible for increased influx of Ca^{2+} resulting in activation of endogenous phospholipases and Ca^{2+} dependent neutral proteases and finally in development of irreversible cell injury.

Deleterious effect of cytosolic calcium accumulation can be partly prevented by calmodulin inhibition. Chlorpromazine (SK + F) 15 mg/kg b.w., or Trifluoperazine (SK + F) 2 mg/kg b.w., administered up to 30 min after ligation of coronary artery were used to achieve full effect.

Treatment of animals with calmodulin inhibitors dramatically improved the ultrastructure of myocytes of the ischemic area and allowed for recovery up to 70 percent of area at risk after restoration of reflow. The membrane protective effect on cardiac myocytes was proved by electron microscopy. Ultrastructural findings correlate with histochemical, physiological and biochemical results.

Advantage of calmodulin inhibition prior to ß-blockers and calcium antagonists in prevention of reperfusion injury consists in the fact that the adverse influence on sarcolemma (receptors and channels) is avoided.

635 IMMUNOCYTOCHEMICAL LOCALIZATION OF APOLIPOPROTEIN B IN THE AORTA DURING PRELESIONAL STAGES OF HYPERLIPIDEMIA, IN RABBIT. Rozalia Mora, Monica Eskenasy, Anton Hillebrand and Nicolae Simionescu, Institute of Cellular Biology and Pathology, Bucharest - 79691, Romania

Hyperlipidemia was induced in male adult Chinchilla rabbits fed a cholesterol - and butter-supplemented diet for time intervals of 1 to 17 weeks. Specimens were collected from lesion prone areas such as the aortic arch and thoracic aorta, and processed by indirect immunoperoxidase technique using a rat anti-rabbit apo B followed by goat anti-rat IgG conjugated with horseradish peroxidase. At 1 week of diet before the appearance of monocyte diapedesis and other detectable lesions, apo B was focally detected both in light and electron microscopy, in the subendothelial space. The reaction product was located extracellularly and progressively accumulated against internal elastic lamina (IE). In the subsequent time intervals, the increasing amounts of apo B paralleled the stromal proliferation and was preferentially associated with the extracellular liposomes (EL) we have previously identified (J.Cell Biol. 1985, 101: 113a). The reaction product appeared either attached to the contour of EL, or forming ~30 to 60 nm particles. Such deposits were not seen in normal animals, and all control experiments (using preimmune serum, goat anti-rat IgG-HRP or DAB alone) were negative. During lesional stages (4-17 weeks), apo B was also found extracellularly in close proximity to foam cells, and against IE. The findings showed that in the prelesional stage there is an increasing extracellular accumulation within the subendothelium of apparently particulate and non particulate apo B preferentially associated with the cholesterol-rich phospholipid lamellae of extracellular liposomes. (Supported by Ministry of Education, Romania, and by NIH Grant HL-26343).

636 VESICULATION CHANGES OF ENDOTHELIAL CELLS OF MICROCIRCULATORY BED IN THE EARLY PHASE OF HAEMORRHAGIC TRAUMATIC SHOCK IN RATS.
Karin Kretschmar, Monika Schiewek. Institute of Pathology, Military Medical Academy; DDR - 1242 Bad Saarow, GDR

A blood volume of 2,5 % of body weight was taken from the carotid artery of male adult R rats narcotized with hexobarbital. Thereafter fracture of posterior extremities was produced. The mesentery of the last ileal loop was taken immediatly, 10 minutes, 20 minutes, 30 minutes and 1 hour after traumatism. After immersion fixation the capillaries were examined by an electron microskope. Morphometric examination of vesicles: double square network, period length 2 mm. Data reprocessing showed in comparison with control animals: Increased volume and surface density, changes of the numeric density of vesicles. Regarding other results, these findings allon the conclusion, that there is an increase of the vesicular transport in the early phase of haemorrhagic traumatic shock.

637 CHANGES IN TISSUE BOUND PLASMINOGEN ACTIVATOR (TPA) IN DISEASE.
U. Fuchs, J. Graff, Leonore Reinknecht, C. Reinknecht. Institute of Pathology. Karl-Marx-University, GDR 7010 Leipzig, Liebigstraße 26, GDR

The proteolytic enzyme Plasmin is activated by the proenzyme Plasminogen which can be activated by tPA. The actual fibrinolytic activity of tissue being the result of pro- and antifibrinolytic tissue factors is proven by fibrinolysis autography done in cryostat sections incubated for 5...60 min at 37°. Veins (varicosis, postthrombotic syndrome, 32 patients), renal tissue (experimental diabetic nephropathy, different degree of metabolic disturbance: average blood glucose 12.5±2.5 and 25.3±2.7 mmol/l resp., 145 rats), lung tissue (experimental virus pneumonia by Myxovirus influenzae A (Leipzig)/1/72 (H_3N_2), 70 rats and mice (a); Cyclophosphamid treatment, 10 rats (b); experimental anthracosis and silicosis, 30 rats (c)) and several normal tissues (blood vessels, lung, kidney, heart, liver, placenta) and ductal carcinoma of breast were morphometrically analysed. The fibrinolytic potential of different tissues is different. Fibrinolytic activity of tissue is proportional to the number of veins and venoles in systemic and to arteries and arterioles in pulmonary circulation. Neighbouring endothelial cells and glomerula may be fibrinolytic active or inactive. In varicosis, diabetic nephropathy, viral pneumonia, silicosis, treatment with cytostatica the fibrinolytic activity is diminished according to the rule: the severer the disease the larger the reduction of activity. This reduction is important for the persistence of fibrin in thrombosis, glomerular deposits, pneumonia and for carcinomatous infiltration. In mechanical damage and after compression of veins the factor is increased. In anthracosis of lung fibrinolytic potential is unchanged. In conclusion substitution and/or increased therapeutical release of tPA may be important in futura

638 ULTRASTRUCTURAL CHANGES IN THE CELLS OF LACTATING MAMMARY GLAND IN MICE AFTER INTRANASAL INFLUENZA INFECTION.

Berezina Nataly. Leningrad Pediatric Medical Institute

The electron microscopic investigation of the cells of the mammary gland in certain stages of pregnancy and lactation after intranasal influenza infection was carried out. During the early stages of influenza infection (2-3 days) the thinning of the endotheliocytes, shortening of the length of specialized contacts between them, the sharp distension of capillary lumen were discovered. Maximum changes in the epithelial cells of the mammary gland were observed on days 4-5 after the infection. The obvious signs of destruction (swelling of the cells, reduction of organelles) were investigated at this stage. In comparison with the normal epithelial cells at the same stages of lactation, the decrease of the elements of the rough surfaced endoplasmic reticulum, free ribosomes, polysomes, secretory material was shown. This reconstruction testifies the decrease of secretory activity of the epithelial cells and their depression caused by influenza infection.

639 DELAYED TOXICITY AND MITOCHONDRIAL EFFECTS INDUCED BY A BIFUNCTIONNAL INTERCALATOR, DITERCALINIUM IN EUKARYOTIC CELLS.

Esnault C., Brown S.C., Roques B.P., and Le Pecq J.B. ; Unité de Biochimie Enzymologie, Institut Gustave Roussy, 94805 Villejuif, France.

The antitumor properties of several 7H-pyridocarbazole dimers, a new series of bifunctionnal intercalators have been described (Pelaprat et al. (1980) J. Med. Chem. 23 : 1336-1343 ; Roques et al., (1979) Biochem. Pharmacol. 28 : 1811-1815). The effects of two dimers (NSC 335153, known as ditercalinium, and NSC 335154) on cell viability, growth, colony formation and cell cycle progression have been investigated on the L1210 cell line and on a L1210 subline resistant to ditercalinium. Several observations indicate that the mechanism of action of these dimers migth be different from that of monointercalating agents : (a) ditercalinium induces a delayed toxicity (growth arrest occuring five generations after drug exposure) in sensitive but not in resistant cells, (b) cells exposed to ditercalinium arrested almost randomly in all phases of the cell cycle, whereas the corresponding monomer provokes a block in the (G2 + M) phase (Esnault et al. (1984), Cancer Research, 44 : 4355-4360). Electronic microscopy examination of cells treated by ditercalinium at dose leading to a delayed toxicity, shows a drastic effect on mitochondria : swollen mitochondria have a clear matrix and the cristae disappeared. The nucleus and the chromatin are not altered. To determinewhether this mitochondrial modification is accompanied by an alteration in the energetic metabolism, the pools of ATP and other nucleotides including NAD were measured by HPLC. No significant variations of these pools are observed before cell lysis.

640 GROWTH, CELL POPULATION HETEROGENEITY AND DNA SYNTHESIS STIMULATION BY AMINOACIDS AND HORMONES OF PRENEOPLASTIC CLONES IN RAT LIVER.

MªDolors Estadella, MªJesús Pujol & Jordi Domingo. Department of Histology and Cell Biology, Faculty of Medicine, University of Barcelona, Diagonal s/n, 08028 Barcelona, Spain.

The development of enzyme deficient foci during chemical carcinogenesis in rodent liver, the clonal origin of such hepatocyte populations and their involvement in the origin of hepatocellular carcinomas has been reported. From our three enzyme studies on serial liver sections and a carcinogenesis protocol including diethylnitrosamine and phenobarbital, we have concluded that foci with more deviated phenotypes grow faster than less deviated ones. The present paper reports the results of four carcinogenesis protocols consisting in one or two cycles of diethylnitrosamine and phenobarbital administration. The phenotype of each focus for the three enzymes (glucose-6-phosphatase, ATPase and 5'-nucleotidase) has been determined by superimposing tracings of serial sections stained for one enzyme. Seven different kinds of foci resulting from simple and combined enzyme deficiencies and also complex foci with smaller foci or subclones inside have been observed. Complex foci appear only in the carcinogenesis protocols with two cycles of diethylnitrosamine and phenobarbital. The number of foci correlates with the duration of the promotion phase (phenobarbital). In the four carcinogenesis protocols the relative proportions of the different foci phenotypes are the same, being the foci deficient only in glucose-6-phosphatase and the foci deficient only in ATPase the most abundant. On the average, complex foci have been found larger than foci without subclones. From comparisons between the number of foci per surface area of liver section and the number of subclones per surface area of focus section, a clear trend that enzyme alterations arise more frequently from cells already modified than from normal tissue can be shown. DNA synthesis in foci and surrounding tissue has been measured in animals stimulated by intravenous infusion of triiodothyronine, aminoacids, glucagon and heparine and compared to non stimulated animals : Basal synthesis is higher in foci than in normal tissue but normal tissue is more stimulable than foci.

641 ANTIGENIC DIVERSION OF RAT LIVER CELLS CHARACTERISTIC OF HEPATOCELLULAR TUMORS AFTER A SINGLE CARCINOGENIC TREATMENT AND PARTIAL HEPATECTOMY. V.A.Ivanov, V.P.Kushner, V.Ja.Fel. Institute of Cytology, Academy of Sciences of the USSR, I94064, Leningrad, Tichoretsky pr.,4, USSR

Antigenic diversion of tumor cell accomplished at the expense of heteroorganic antigens is a most typical break in the cytodifferentiation at malignization. The synthesis of kidney heteroorganic antigens has been studied in rat liver after treatment with hepatocarcinogens - 4-dimethylaminoazobenzene (DAB) and N-nitrozodiethylamine (DENA). The antigens were revealed by means of immune serum against non histone proteins (NHP)-DNA complexes from the kidney of intact rats in the composition of free from DNA preparations of NHP chromatin from rat liver I-I2 and I-64 days after DAB and DENA treatment, respectively and what is important, 4 days after partial hepatectomy.Using immune serum of narrow specificity against membraneous heteroorganic antigens of kidney associated with Zajdela hepatoma cells the above antigens at the hepatocyte surface isolated from rat liver after single treatment with DAB or DENA or after partial hepatectomy were described quantitatively. The synthesis kidney membrane heteroorganic antigens was registred in thes case within 2I, 60 and II days, respectively. The maximum was attained on the 4 day when thecontent of antigens reached that of characteristic of Zajdela hepatoma cells. The presence in the nuclear NHP and on the membranes of hepatocytes of heteroorganic antigens of the same tissue nature indicates relationships between the both phenomena ander analysis.

642 CHARACTERIZATION OF HUMAN OR RAT NORMAL AND NEOPLASTIC CELLS IN VITRO BY DIFFERENT METHODS. E. Nissen (1), W. Lehmann (2), W. Arnold (1), St. Tanneberger (1). Academy of Sciences of the GDR, (1) Central Institute of Cancer Research, (2) Central Institute of Molecular Biology, DDR - 1115 Berlin, Lindenberger Weg 80, German Democratic Republic (GDR)

Several human or rat normal and neoplastic cells of the breast, liver, ovary and endometrium were cultivated. These cells were characterized by flow cytometry growth in soft agar and multinucleation by cytochalasin B treatment. The results of these in vitro methods were compared with transplantation in vivo. Neoplastic cells of both species provide tumours in vivo and can be characterized in vitro by changed DNA-distribution pattern (hyperdiploid-polyploid), three-dimensional growth in soft agar and more than 3 nuclei per cell. Normal cells did not show such properties with exception of rat liver cells. These cells demonstrated a hyperdiploid DNA-distribution pattern but no growth in soft agar. The transplantation experiment was negative. Are these cells preneoplastic ?

643 CORRELATION BETWEEN VIRUS SURFACE ANTIGENS AND SUBMEMBRANOUS VIRUS STRUCTURES BY DOUBLE IMMUNOLABELING. EM INVESTIGATION OF PROTOPLASMIC SURFACE REPLICA.

Gabriel Rutter, Heinz Hohenberg, Wolfgang Bohn, Klaus Mannweiler. Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie, Martinistr.52, 2000 Hamburg 20, Fed. Rep. Germany.

The maturation of enveloped viruses as models provides valuable insights into the organization of plasma membrane (PM). For instance, measles virus (MV) particles assemble at the PM and are released from the cells by a budding process. In a previous work (Rutter et al., Eur. J. Cell Biol. 1986) we could correlate the viral structures seen at the protoplasmic surface (PS) with antigenic determinants present on the exterior of the cell surface itself. To further characterize the morphological events accompanying the assembly of MV at the PM, we developed a method permitting the concomitant immunocytochemical identification of antigenic structures on both sides of the PM on the same replica. Virus antigens at the apical cell surface were labeled with rabbit anti MV antiserum and protein A gold. The apical PM were isolated and labeled on the PS with different mouse monoclonal antibodies against MV capsid polypeptides by the PAP method. The critical point dried PM were shadowed with platinum/carbon and replicated. With this method we could demonstrate the polypeptide composition of MV structures at the PS and its correlation to external PM antigens in the course of infection.

644 ELECTRON MICROSCOPIC-IMMUNCYTOCHEMICAL STUDIES ON POLIOVIRUS BINDING SITES AT VERO CELLS BY MONOCLONAL ANTIBODIES.

Klaus Mannweiler, Peter Nobis*, Heinz Hohenberg, Wolfgang Bohn.
Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie a.d.Univ.Hamburg and Abteilung Molekularbiologie, Univ.Hamburg*, D-2000 Hamburg 20, Fed. Rep. Germany.

Specific receptors are responsible for the attachment of poliovirus to the surface of susceptible cells. We studied the three-dimensional distribution of this cellular receptor by immunogold labeling and replica-technique (Mannweiler et al., J.Microsc. 1981, Vol. 126, 145-149) using a monoclonal antibody against the poliovirus receptor (Nobis et al., J.Gen.Virol. 1985, 66, 2563-69). Vero cells labeled with this antibody and with protein A gold either prior to or after fixation showed clusters of 3-7 gold particles at the cell surface. Only a few isolated immunomarkers were visible on these cells after labeling with nonspecific antibodies. The pattern of IM granules at the labeled areas were studied in labeled-freeze-fracture preparation of the plasma membrane (H.Hohenberg et al., in press, Science Biol.Specimen. Prep., SEM Inc., O'Hare, Chicago, 1986). The distribution of the receptor correlates with that of poliovirus adsorbed to the cell surface at 4° and thereafter labeled with rabbit anti-poliovirus-antibodies. The initial steps of poliovirus entry were further studied on labeled and freeze-fractured plasma membranes of infected cells. Adsorption leads to a redistribution of IM granules. Internatization of the virus is characterized by the appearance of membrane invaginations at the protoplasmic fracture face.

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645 Ca^{2+}-INDUCED DISASSEMBLY OF BUDDING MEASLES VIRUS AT THE PLASMA MEMBRANE

Wolfgang Bohn,Gabriel Rutter, Heinz Hohenberg, Klaus Mannweiler. Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie,Martinistr.52, 2 Hamburg 20, Fed.Rep.Germany

Measles virus completes its morphogenesis by an assembly of the structural components at the plasma membrane. Recently we could demonstrate that actin filaments emanate into budding virus with the barbed ends indicating that budding is connected with a polar growth of these filaments(Bohn et al.,Virology in press,1986).Furthermore we could show that budding can reversibly be arrested at a distinct step by phenothiazines (Bohn et al.,Virology 130,44-55,1983). These data led us to assume that budding of measles virus might be regulated by Ca^{2+}-ions. As we now demonstrate an increase of the intracellular calcium concentration induced by the calcium ionophore A23187 disrupts assembled virus structures at the plasma membrane and leads to a uniform distribution of viral envelope proteins in the plane of the plasma membrane. This effect is strictly dependent on the presence of Ca^{2+}, and can not be induced by Mg^{2+} or Ba^{2+}. Pt/C-replicas of isolated apical plasma membranes prepared from these cells show that viral nucleocapsids, which are normally detectable at the inner side of the plasma membrane , are absent. Viral antigens at the plasma membrane can completely be extracted from these cells with non-ionic detergents, indicating that their linkage to the cytoskeleton is disrupted. The data support the idea, that Ca^{2+}-ions regulate both the assembly as well as the budding of at least this virus.

646 FREEZE FRACTURE MORPHOLOGY OF INTERACTIONS BETWEEN INFLUENZA VIRUS AND LIPOSOMES DURING MEMBRANE FUSION.

G. Knoll(1), G. van Meer(2), A.J. Verkleij(1). (1) Institute of Molecular Biology, State University of Utrecht, Padualaan 8, NL-3584 CH Utrecht, The Netherlands, and (2) EMBL, Meyerhofstr. 1, D-6900 Heidelberg, FRG

Influenza virus-particles and liposomes can be induced to fuse by lowering the pH of the incubation medium. This was shown by assaying either the mixing of the aqueous compartments (1) or the mixing of the phospholipids (2) of the virus and the liposomes. The membrane fusion is mediated by a viral envelope-protein and represents probably the best characterized biological fusion system at a molecular level (3). But the precise mechanism of the actual joining of the lipid bilayers is still unknown. The morphology of membrane fusion events has been shown to be highly sensitive to artefacts induced by chemical pretreatments (4). Therefore pure physical fixation by rapid freezing was applied. Liposomes containing appropriate gangliosides as receptors (essentially as described in (5), and influenza virus-particles were allowed to bind at neutral pH. For the induction of fusion the pH was lowered to pH 5. Samples were frozen in both conditions by thin-sandwich propane-jet freezing (6). An assembling device built for this purpose makes it possible to lower the pH of the suspension and freeze it synchronously thereby allowing to catch very early fusion stages. The use of a nitrocellulose-filter sheet as part of the sandwich permits to freeze fracture the thin sample at a right angle to its general plane thereby allowing the localization of structures relative to the plane of the sandwich. Distinct morphological features of the binding and fusion stages will be shown and possible relevance for the understanding of the fusion mechanism discussed. (1) White, J. et al. (1982), EMBO J. 1:217-222. (2) Stegmann, T. et al. (1985), Biochemistry 24:3107-3113. (3) White, J. et al. (1983), Quart. Rev. Biophys. 16:151-195. (4) Plattner, H. (1981), Cell Biol. Int. Rep. 5:435-459. (5) van Meer, G. et al. (1985), Biochemistry 24:3593-3602. (6) Knoll, G. et al. (1982), Protoplasma 111:161-176.

647 TRYPSIN EFFECTS ON LYTIC ACTIVITY OF SENDAI VIRUS.

Nicola MOSCUFO, Angelo Gallina, Maurizio Tomasi. Lab. BIOLOGIA CELLULARE Istituto Superiore di Sanità Viale Regina Elena, 299 00161 ROME, ITALY

Sendai Virus (SV) possesses an envelope able to fuse with the membrane of recipient cells. This property is due to the presence of two glycoproteins HN and F. The HN binds the virus particles to cell surface and together with F induces fusion. It is known that trypsin digestion of native SV produces selectively two F peptides (32000 and 19000 daltons.) Effects of trypsin treatment on SV have been followed either by PAGE patterns in SDS or by disappearing of fusogenic activity, this, in turn may be extimated by lysis virus-induced on human erythrocytes (HE). Trypsin digestion of SV for 30' at 37°C as 120' at 25°C as 8 hs at 6°C produced 90% decreasing of lytic activity. By contrast the binding, measured by haemagglutination test, resulted unaltered. Interesting we observed that trypsin may also produce an activating effect on SV lytic capacity. The activation was maximal when trypsin was incubated with SV at 4°C for 5', then HE were added and the mixture were incubated for 10' at 25°C. Moreover we observed that the presence of trypsin at 25°C produced a rate of lysis very similar to that obtained by condition without protease at 37°C. No enhancing of lysis was detected when trypsin treatment was performed separately on HE and SV. Although the molecular mechanism of this phenomenon is quite obscure we notice that at 25°C trypsin promotes on interacting SV with HE and viceversa some processes which normally occur at 37°C.

648 LEAVES AND ROOTS OF MAIZE PLANTS INFECTED WITH BARLEY YELLOW DWARF VIRUS (BYDV).

Maria Augusta Favali(1),Ruggero Osler (2) and Paola BETTINI (1).(1)Department of Biology,University of Padova,Via Orto Botanico 15,35123 Padova,and (2)Plant Protection Institute,University of Udine.Italy.

In a previous paper (Favali et al.,Mitteilungen aus der Biologischen Bundesanstalt,228,21-26,1985), we reported the first results of ultrastructural studies and X-ray microanalysis carried out on leaves of maize plants infected with BYDV,a typical member of the luteovirus group.BYDV has been proven to be a cosmopolitan pathogen,existing in several strains that vary in insect vector specificity and causing economic losses in several cereals.In maize,the disease is characterized by stunting of the plant and dark-red colouration of the leaves.By electron microscopy (E.M.),we observed in infected cells,many alterations:as an example a deeply abnormal accumulation of starch granules in the plastids of the bundle sheath cells and inclusions bodies,crystalline or amorphous,different in size and shape,localized in the phloem cells.Because of the great interest about these structures,we studied in more details both leaves and roots (never observed before) of different lines in order to compare the inclusions bodies in both tissues by E.M. techniques and by analysis of micrographs ,recorded at a 40,000 magnification and digitized according the method described by De Rosier and Klug(Nature,217,130-134,1968).From our results it seems that the genotype of each line of maize tested may interact with the infecting BYDV to cause many alterations,some of them peculiar for each line.Therefore a better knowledge of their fine structure and composition seems fundamental to an understanding of their probable role in the infection process.

649 O_2^--GENERATING OXIDASE - THE BASIS OF LYMPHOCYTE RADIOSENSITIVITY? MARGERY G. ORD, LLOYD A. STOCKEN, DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF OXFORD, SOUTH PARKS ROAD, OXFORD, OX1 3QU, U.K.

A plasma-membrane associated, O_2^--generating oxidase of rat thymocytes was 40% inhibited in cells exposed to 4 Gy in vitro. If glycolysis was inhibited with 4 mM iodoacetamide to enhance glucose utilisation by the pentose-phosphate route, 90% inactivation was produced by 4 Gy. The radio-sensitive step was the cytochrome b-associated superoxide production, in small lymphocytes. It is suggested that in these highly specialised small cells, essential functions may be dependent on nuclear phosphorylation and the oxidase, through the following reactions:

1) $3\ \text{G-6-P} + 6\ \text{NADP}^+ + 5\ \text{NAD}^+ + 5\ P_1 + 8\ \text{ADP}$
$5\ \text{PYRUVATE} + 5\ \text{NADH} + 3\ CO_2 + 8\text{ATP} + 6\ \text{NADPH} + 8\ H^+$

2) $6\ \text{NADPH} + 12O_2 \quad 6\ \text{NADP}^+ + 12O_2^- + 6\ H^+$,

NADH being recycled to NAD^+ because of the concomitant presence of lactic dehydrogenase.

650 ULTRASTRUCTURAL DEMONSTRATION OF AMINE- AND CHOLERATOXIN-STIMULATED ADENYLATE CYCLASE IN RAT CEREBRAL CORTEX AND BROWN ADIPOSE TISSUE.
L. Rechardt (1) and H. Hervonen (2). (1) Department of Biomedical Sciences, University of Tampere, Box 607, 33101 Tampere, Finland (2) Department of Anatomy, University of Helsinki, Siltavuorenpenger 20, 00170 Helsinki, Finland.

Adenylate cyclase (EC 4.6.1.1.) enzyme is one of the key enzymes in research on the receptor-mediated regulation of the cell function. We have studied the cytochemical localization of this enzyme activity by using as a trapping agent cobalt (Histochemistry 51:113, 1977) or cerium (Histochemistry 82:82, 1985). The tissues were the cerebral cortex of adult rats and brown adipose tissue of new-born rats. The enzyme activities were stimulated by choleratoxin, amines or amine-agonists and blocked by beeta-blockers both in vivo or in vitro. The cerium ion yielded by far the most finely crystalline and easily visible precipitate which in the X-ray microprobe analysis revealed cerium and phosphate peaks, respectively.

The cytochemical reaction product was observed in the synaptic profiles in several types of localizations: 1) only in the postsynaptic membrane-thickening, 2) in the postsynaptic thickening and profile, 3) in the presynaptic nerve terminal, 4) both in the postsynaptic profile and presynaptic nerve terminal.

In the stimulated adipocytes of the newborn rats the reaction product was seen attached into the cell membrane in the plasmalemmas.

Especially the use of cerium ion offers a new tool, valid tool for cytochemical localization of adenylate cyclase enzyme related to the membrane receptors.

651 **ADENYLATE CYCLASE ACTIVITY IN CELLS INVOLVED IN GONOCOCCAL INFECTION.**
G.A.Dmitriev (I), I.B.Buchwalow (2). (I) Central Research Institute for Skin and Venereal Diseases, I07076-Moscow, Korolenko str., 3, and (2) All-Union Research Centre for Mother and Child Health Protection, II75I3-Moscow, Oparin str., 4, USSR

Altered levels of cyclic AMP in the cytoplasm elicit the specific cellular responses once attributed to the action of hormones exclusively. Assuming it to be a universal mechanism for both eu- and prokariotic cells, we have undertaken an ultracytochemical study of adenylate cyclase activity in leucocytes and gonococci from the urethral discharge of untreated patients and in cultured gonococci raised on a solid nutritive medium. Ultracytochemical demonstration of adenylate cyclase activity was carried out according to a modified technique of Wagner and Bitensky (Buchwalow et al., Histochemistry, I98I, v.72, p.625-634).

Adenylate cyclase activity was detected in cultured gonococci on the inner side of the cytoplasmic membrane, in the regions of ribosome concentration and in the nucleoid. The enzyme activity was markedly enhanced with IO mM NaF especially in the cytoplasmic membranes in the sites of partition of dividing bacterial cells, which might be accounted for by an increased turnover in the cell division process. In leucocytes from the urethral discharge of untreated patients, activity of fluoride-sensitive adenylate cyclase was demonstrated on the cytoplasmic side of the plasma membrane.

Further investigations on leucocytes and microbial cells from treated patients and on cultured gonococci strains with different sensitivity to antibiotics are in progress.

652 PURIFICATION AND CHARACTERIZATION OF LIPID SENSITIVE, Ca-DEPENDENT PROTEIN KINASES FROM LUCERNE. Z.Olah (1) and L.Bögre (2). (1) Institute of Biophisics, (2) Institute of Genetics, Biological Research Center, Hungarian Academy of Sciences, H-6701 Szeged, POB 521, Hungary.

Cells of lucerne (Medicago varia L.cv.Rambler) suspension culture were fractionated by differential centrifugation and a lipid sensitive, Ca-dependent protein kinase was purified. The enzyme was recovered from the 30,000 g supernatant by DEAE-cellulose chromatography and separated to further two fractions by gelfiltration on Sephacryl S-200. The kinase of lower molecular rate needed lisine rich protein (H1 histone) as a substrate for its activity, therefore, it was purified near to homogenity by affinity chromatography on histone-Sepharose column. To find out Ca-depending lipid sensitivity and phosphorylation properties of the purified enzyme two dimensional gel electroforesis was applied. Labelling with (γ-^{32}P) ATP of 30,000 g supernatant proteins as substrates revealed several other endogenous targets of the enzyme. Several protein phosphorylated only in the presence of Ca and phosphatidyl serine. Our data suggest the occurance of lipid sensitive, Ca-dependent phosphorylation system in lucerne cells, which might take part in transmembrane signalling similar as it is performed in animal cells.

653 STUDY ON ALKALINE PROTEASES OF THE SILKWORM GUT CELLS. M. Eguchi[1] and K. Kuriyama[2] 1) Kyoto Institute of Technology, Matsugasaki, Kyoto, Japan, 2) Nihon Nohyaku Co. Ltd., Kawachi-Nagano, Osaka, Japan.

Alkaline proteases are contained in the midgut lumen and epithelia of the silkworm, Bombyx mori and most of the tissue proteases are membrane bound enzyme. The digestive fluid proteases were separated into 3 fractions, 6B1-3 on a Sepharose 6B column and the 6B3 was the most abundant of 3 proteases. On the other hand, these lumenal proteases are considered to be secreted from the midgut epithelial cells. We studied the relationship between proteases from the midgut lumen and epithelia.

When the midgut epithelial protease was solubilized with Lubrol WX under neutral condition, the protease 6B2-T corresponding to the digestive fluid protease 6B2 was obtained, whereas under alkaline condition the protease 6B3-T was produced. In this case, marked increases of solubilization and activity were observed at higher than pH 8; the proteolytic activity was enhanced more than 2.3 times at pH 12.0.

These results suggests that the 6B2-type protease produced in the midgut epithelium under neutral condition would be transfered and discharged into the gut lumen, and converted into the main protease-6B3 under alkaline conditions.

Two purified proteases, caseinolytic (6B3-Tc) and benzoyl-arginine-p-nitroanilidelytic (6B3-Tb) were obtained from the midgut tissue. These enzymes showed high pH optima, 11.2, and pI values, above 11, and were extremely stable over a wide range of pH, especially under alkaline conditions.

654 APPLICATION OF WHEAT CHLOROPLAST NUCLEASE TO THE STRUCTURAL STUDY OF NUCLEIC ACIDS. Elżbieta Kuligowska, Anna Przykorska, Danuta Klarkowska, Jan W. Szarkowski. Institute of Biochemistry and Biophysics, Polish Academy of Sciences, PL-02-532 Warszawa, Rakowiecka 36, Poland.

A sugar nonspecific nuclease from wheat chloroplasts shows specificity towards single-stranded DNA and RNA and can be applied to structural studies of nucleic acids. The nuclease can recognize the relaxed secondary structure of DNA and attacks covalently closed, circular DNA of plasmids ColE1 and pBR322. The enzyme converts them initially into the open-circular form and subsequently into the linear form. Single-stranded Φ X174 DNA is also hydrolysed by the enzyme. The reaction is not random and the nuclease cuts Φ X174 DNA at four specific sites as detected by electrophoresis of the reaction products on polyacrylamide gels and scanning them at 260 nm. The enzyme selectively attacks single-stranded regions in small RNA molecules possessing a high degree of secondary structure.

655 THE GENES ARE NOT BATHING IN A 150 mM ELECTROLYTE SOLUTION IN THE NUCLEUS OF A LIVING CELL. Miklós Kellermayer, Department of Clinical Chemistry, University Medical School, Pécs, 7624 Hungary.

Previously, a stained polarization optical technique invented by Gy.Romhányi has been used as a sensitive "tool" to study the inorganic ion induced structural changes of DNA inside the chromatin structures of the cell nucleus. Furthermore, this technique allowed us to recognize that DNA in the living cell nucleus can not be exposed to a 150-200 mM free electrolyte solution which has been supposed to be characteristic for the ionic milieu of the nucleus and the cell as well. The isotropic appearance of DNA inside the nucleus of an intact cell requires a mechanism which decreases the concentration of the free cations and prevents the DNA from the unfolding effect of the inorganic ions. The experimental results we are going to present indicate that the DNA becomes anisotropic at the moment of the cell death, while at the agony of the cell, the nucleus shrinks or becomes pyknotic. The nuclear pyknosis and the concomitant optical anisotropy of DNA indicate that inside the cell nucleus a sequential increase in the ratio of the free ions takes place during the process ending up with the death of the cell. Findings on isolated nuclei also indicate the close relationship between the volume changes of the nuclei, the isotropic-anisotropic conversion of DNA and the ion concentrations around the nuclei. The involvement of the "loosely bound" proteins and water molecules in regulation of the ionic milieu and the structural characteristics of DNA within the living cell nucleus is going to be discussed.

656 OSMOTIC PROPERTIES OF CELLULAR WATER. Ivan L. Cameron[1], Philip J. Merta[1] and Gary D. Fullerton[2], Departments of Cellular and Structural Biology[1] and Radiology[2], The University of Texas Health Science Center at San Antonio, San Antonio, TX 78284 U.S.A.

Cells do not act as perfect osmometers which may be accounted for by an "osmotically inactive" water fraction. Recent NMR titration experiments have identified a hydration water compartment associated with hydrophilic macromolecules. We hypothesized that this hydration water in cells is "osmotically inactive" compared to bulk water. To test this hypothesis we collated literature values and made independent measurements on the extent of hydration water as well as on the extent of "osmotically inactive" water on several specimens. Hydration water measurements were made by a recently introduced pulsed proton NMR titration procedure (Fullerton et al., 1986) or by a new ice crystal size method (Cameron et al., 1986). Osmotically inactive water determinations were made by exposing the specimens to solutions of different osmotic pressure. The following is a listing of the extent of hydration water and the extent of osmotically inactive water both in g H_2O/100 g dry mass. Unfertilized sea urchin eggs, 128 and 131; Artemia brine shrimp cysts, 140 and 116; mammalian lens, 128 and 117; frog muscle, - and 120; fully grown Xenopus oocytes, - and 118, human hemoglobin, 126 and 142; lysozyme, 141 and -. Fertilization of the sea urchin eggs caused a decrease in hydration water from 128 to 56 g/100 dry mass but we have yet to determine if a parallel change occurs in the "osmotically inactive" water. The hydration water values and the "osmotically inactive" water values are similar in each specimen and the mean values of all specimens do not differ significantly. We conclude that under physiological condition hydration water is restricted in motion such that it does not participate in the ideal osmotic equation of van't Hoff. In other words hydration water appears to be "osmotically inactive".

657 WHAT DISTINGUISHES A LIVING CELL FROM A DEAD ONE? Gilbert N. Ling, Ph.D., Molecular Biology Department, Pennsylvania Hospital, 8th and Spruce Streets, Philadelphia, PA 19107, USA

The successful rearing of a human baby from a fertilized, once deeply frozen ovum offers additional suggestive evidence that at the cell level, being alive signifies the maintenance of a static (rather than dynamic) state, given the name, the " living state", in the association-induction (AI) hypothesis.

The living state is a high energy, low entropy state. In this state the three major components of the cell, proteins, ions, and water are closely associated: the major cell cation, K^+, is adsorbed singly on β- and γ-carboxyl groups while the bulk of cell water is adsorbed in multilayers (on some fully extended polypeptide chains) with reduced solubility for large molecules and hydrated ions like Na^+. In dead cells, K^+ and water become liberated, the β- and γ-carboxyl groups are locked in salt linkages and the backbone NHCO groups, internally neutralized forming intra- and intermacromolecular H-bonds.

The existence of the descrete living and dead states rests upon the (auto)cooperativity in the interaction between protein and protein, between protein and water, and between protein and ions. This cooperativity, in turn, is the consequence of nearest neighbor interaction among protein sites, primarily electronic and inductive in nature. The maintenance of the living cell requires the interaction of the protein with key controlling agents, collectively called cardinal adsorbents (CA), including ATP, drugs, hormones, etc. The binding of a cardinal adsorbent onto a receptor site on cell proteins, may draw electrons toward itself (an electron withdrawing CA or EWC), donate electrons (an electron donating CA or EDC), or does neither (an electron indifferent CA or EIC). ATP appears to be an EWC. When adsorbed, ATP causes a propagated decrease of the electron-density or c-value of the β- and γ-carboxyl groups, increasing their preference for K^+ over Na^+, for example.

Removal of ATP causes redistribution of electrons in the protein-water-ion system and in consequence, a conformation change as during some type of cell activation.

658 POTASSIUM BINDING AT CELLULAR PROTEIN SITES VISUALIZED BY THALLIUM IN FROZEN HYDRATED CRYOSECTIONS. L. Edelmann, Medizinische Biologie, Fachbereich Theoretische Medizin der Universität des Saarlandes, D-6650 Homburg/Saar, FRG.

Living cells accumulate K^+ ions selectively and partly exclude Na^+ ions. The commonly taught membrane theory maintains that membrane situated pumps are responsible for the asymmetric ion distribution; it is assumed that virtually all cellular K^+ is freely dissolved in free cellular water. In the alternative association induction hypothesis the asymmetric ion distribution is dependent on selective ion adsorption to cellular proteins and specific physical properties of cellular water; the bulk of cellular K^+ is adsorbed on β- and γ-carboxyl groups of cell proteins. With the following established facts an unequivocal test of the alternative views of the physical state of cellular K^+ is possible: 1) Tl^+ is accumulated in the living cell by the same molecular mechanism as K^+; cellular $K^+$of living frog muscle can be replaced by Tl^+ in a mole-for-mole fashion. 2) An uneven distribution of the electron dense Tl^+ in a thin tissue section can be directly visualized in an electron microscope. 3) Frozen hydrated cryosections can be investigated in a cryo electron microscope; density differences within the sections are directly visible. Hence, it can be tested if Tl^+ in a frozen hydrated muscle cryosection follows the water distribution or if it is accumulated at those proteins which are expected to bind K^+.

Tl^+ loaded frog sartorius muscles have been cryofixed and cryosectioned. Frozen hydrated sections have been examined in a ZEISS EM10CR. The sections revealed Tl^+accumulation at protein sites, mainly at myosin, at actin and at Z-line proteins. The Tl^+ accumulation pattern resembles the staining pattern of conventionally prepared and stained muscle sections. The results support the association induction hypothesis.

659 THE ACTIVATION OF METABOLISM BY WATER IN DRY BIOLOGICAL SYSTEMS. James S. Clegg, University of California, Bodega Marine Laboratory, Bodega Bay, CA 94923, USA.

Most cells die when severely dehydrated. Presumably, this forms the basis for the apparent conclusion that water is essential for life, as we know it at least. The details of how and why water plays this vital role are, however, not well defined. It is of interest, therefore, that certain organisms have acquired the ability to reversibly lose practically all of their intracellular water. Such organisms are commonly referred to as "cryptobiotic" since, when severely dried, they appear to be neither alive nor dead when evaluated by commonly adopted criteria: metabolism is brought to a standstill, thereby halting all the dynamic aspects exhibited by living cells. The cryptobiotic state, therefore, is a unique level of biological organization and is of fundamental importance itself. In addition, however, such organisms allow us to explore a variety of biological questions. The one to be examined in this presentation will be the initiation of metabolism by addition of water to dried cysts of the brine shrimp Artemia. This system has been the object of considerable research, and its properties and water-dependent activities will be summarized. A general "model" of the aqueous compartments of animal cells has been developed from this research and will be presented. It indicates that water may not simply allow metabolism to occur, but may play an essential role in its organization and regulation.

660 INTERACTIONS OF WATER MOLECULES WITH THE CYTO- AND NUCLEAR MATRICES OF THE CELLS. C.F. Hazlewood, Baylor College of Medicine, Houston, Texas (USA) 77030

It is well known that the diffusive motion of water within cells is such that, when one assumes simple brownian motion, each molecule can experience the entire volume of several cells within one second. Nevertheless, the diffusive motion of water in cells, in tissues, and even in organisms has been shown to be reduced relative to that of ordinary bulk water or water in dilute ionic solutions. Furthermore, this reduction in diffussive motion has been found to involve rotational as well as translational motion and that the reduction observed is present after corrections for obstructive barriers. That is, there seems to be an intrinsic slowing of the motion of the water molecules due to the interaction of the water molecules with the macromolecular structures within the cells.

The reduction in rotational and translational motion of water in cells alone, however, is insufficient to account for other changes in the physical properties of water that occur in cells and tissues. For example, there is a relatively weak frequency dependence of the nuclear magnetic resonance (NMR) relaxation time, T_1, of water protons in cells and tissues. Additional mechanisms, therefore, must be sought to explain the observations. Using conventional NMR theory of relaxation in liquids, T_1 of protons is expected to vary as the square of frequency (ν^2). In most cells and tissues the T_1 has been found to vary more nearly as the $\nu^{1/2}$. We have considered a single chain model for a polymer protein (Rouse, P.E., J. Chem. Phys. 21:1273, 1953) and derived equations that closely describe the observed behavior of the T_1 with frequency. The theory proposes that the frequency dependence of the relaxation times of water protons in biological systems is associated with the motional fluctuations of protons macromolecules (probably proteins). These protons may be present as hydration water tightly bound to the polymer, or as freely exchangeable covalently bonded side-groups of proton pairs. If this theory proves to be correct, then the relaxation of water protons will be determined by not only the bulk properties of water; but also, by the interaction of water with the macromolecular excitations of the proteonaceous matrices of the cell.

661 MNR STUDY ON THE INFLUENCE OF SOME CYCLOOXYGENASE AND THROMBOXAN SYNTHETASE INHIBITOR ON TISSUE WATER.

M. Nechifor (1), I. Baciu (2). (1) Dept. of Pharmacology, and (2) Dept. of Biophysics, Medical School, 6600 Iasi, Romania

The influence of phenylbutasone (50 mg/kg i.p.), indomethacin (20 mg/kg i.p.), imidasole (100 mg/kg i.p.), each administered separately to a series of adult, white, male rats was studied by impulses MNR . The tests were performed on liver, lung and kidney collected from decapitated rats.

A MNR device type AREMI 78, and a personal programme in Basic were applied.

The tests were performed 60 minutes after the administration of the cyclooxygenase or thromboxansynthetase inhibitors.

The obtained results point onto a maximum MNR relaxation time significantly shorter in the case of the series treated with phenylbutasone (17-19 ms) and indomethacin (18-20 ms) as compared to the control series (95-47 ms). Imidasole also shortens the relaxation time (from 45 to 31 ms in the lung) a maximum MNR being obtained.

These data plead for an action of these substances on tissue water and lead to the opinion that the prostaglandins and thromboxans play a role in the cytoplasmatic water states.

A fourth series of animals, bread under similar conditions, served as controls.

MNR = magnetic nuclear resonance

662 ON THE ROLE OF POLYAMINE SYNTHESIS IN PHENOTYPIC MODULATION OF ARTERIAL SMOOTH MUSCLE CELLS

Johan Thyberg (1), Bertil B. Fredholm (2). (1) Department of Histology and (2) Department of Pharmacology, Karolinska Institutet, S-104 01 Stockholm, Sweden

During in vitro cultivation arterial smooth muscle cells go through a transition from contractile to synthetic phenotype. Morphologically, this process includes partial regression of the myofilament system and formation of an extensive rough endoplasmic reticulum and a large Golgi complex. Functionally, it leads to that the cells stop contracting and instead gain the ability to synthesize DNA, divide and secrete extracellular matrix components. Here, we have examined the role of polyamines in the phenotypic modulation of adult rat aortic smooth muscle cells in primary culture. Ornithine decarboxylase, the major rate-limiting enzyme in polyamine synthesis, showed no detectable activity in freshly isolated contractile cells, raised to a peak in activity on day 2-3 and then returned to low levels on day 5-7 when the cells became confluent and ceased growing. This induction of ornithine decarboxylase activity was accompanied by a prominent increase in the cellular concentrations of the polyamines putrescine, spermidine and spermine. Treatment of the cultures with α-difluoromethylornithine, an inhibitor of putrescine synthesis, slowed down the rates of transition into synthetic phenotype (as assessed electron microscopically) and cellular proliferation. Combined treatment with α-difluoromethylornithine and methylglyoxal-bis(guanylhydrazone), an inhibitor of spermidine and spermine synthesis, retarded the transition into synthetic phenotype further and completely blocked cell growth. The results indicate that activated polyamine synthesis plays an important role in the modulation of arterial smooth muscle cells from contractile to synthetic phenotype as well as in the resulting cellular proliferation.

663 PRESERVATION OF PHENOTYPIC TRAITS IN CULTURED BREAST EPITHELIAL CELLS WITH PARTICULAR REFERENCE TO POLARIZED FEATURES.

Ole William Petersen and Bo van Deurs. Department of Anatomy, The Panum Institute, Blegdamsvej 3, DK 2200 Copenhagen N, Denmark.

We have used a short-term, serum-free monolayer culture system to maintain epithelial cells with preserved phenotypic traits from human breast carcinomas. Several morphological and enzyme- and immunocytochemical characteristics of carcinoma epithelial cells (CEP) and of normal lining epithelial cells (LEP) and myoepithelial cells (MEP) were defined in vivo based on cryosections of biopsy material and used to identify cells in the derived primary monolayer cultures. In normal breast ductules, LEP and MEP showed a high degree of polarity. Thus, LEP was characterized by an apical surface with a brush border and expressing two epithelial specific antigens, and more cytokeratins than seen in MEP. MEP, in contrast, rested on a basement membrane, detected by type IV collagen immunocytochemistry. These differentiated patterns were preserved in culture, where MEP formed the outer part of the epithelial islets, while LEP comprised the central part of the monolayer. MEP proliferated with PDTs of ca. 1 day, and LEP of ca. 3 days, as measured by computerized image analysis. A population of CEP could be distinguished from LEP and MEP in vivo by reaction for the enzyme NADPH-NT reductase. Some monolayer cell islets in the derived cultures also showed distinct reaction for this enzyme. CEP in vivo as well as in vitro also showed marked reaction for the pentose-shunt enzyme glycose 6-phosphate dehydrogenase. In contrast, LEP/MEP islets showed no reaction for either enzyme. The CEP islets expressed both the LEP surface antigens as well as cytokeratins, although with marked heterogeneity or modulations, whereas cell-associated type IV collagen could not be detected. Using DNA fluorimetry it was shown that some of the CEP islets were aneuploid, and showed no measurable proliferation, while diploid CEP islets sometimes proliferated with PDTs of ca. 5 days. Finally, electron microscopy of CEP revealed some degree of apparent polarity, and frequent intracytoplasmic lumina.

664 AN IN VITRO ANALYSIS OF HUMAN PANCREATIC DUCT EPITHELIAL CELLS.

Ann Harris and Lindsay Coleman. Paediatric Research Unit, United Medical and Dental Schools of Guy's and St. Thomas's Hospitals, University of London. ENGLAND.

An in vitro tissue culture system is being set up for human pancreatic duct epithelial cells. The aim is to investigate the biochemistry and molecular biology of these cells, both from normal individuals and cystic fibrosis patients. Fetal pancreas from mid-trimester terminations is being used as a source of these cells.

Data will be presented on the methods of establishing human pancreatic duct epithelial cells in culture and on their nutrient requirements for continued growth and replication. Specific morphological, biochemical and immunocytochemical characteristics of these cells will be described.

665 INNERVATION OF CHICKEN EMBRYONAL MYOTUBES GROWN IN A SERUM-FREE MEDIUM.
M. Tolar, J. Michl, H. Zemková, J. Teisinger, J. Štichová. Institute of Physiology, Czechoslovak Academy of Sciences, Vídeňská 1083, 142 20 Prague 4, Czechoslovakia.

Combined cultures of nerve and muscle tissue represent an important model for studies concerned with neuro-muscular relationships. Such studies have been carried out in cultivation media containing whole serum supplement, thus introducing poorly defined factors into this system. Replacement of the serum by its one protein fraction (growth-promoting alpha-globulin, GPAG, M.Tolar et al., Mol.Physiol. 3 (1983) 151-166) not only simplified conditions, but also improved results of cultivation.

Dissociated chicken embryonic muscle cells were cultivated in Eagle's minimal essential medium supplemented with foetal calf serum (with or without chicken embryonal extract) or GPAG isolated from calf serum. GPAG promoted multiplication of myoblasts, their fusion, differentiation of myotubes to spontaneous contractility and innervation of the myotubes by dissociated spinal cord neurones. In comparison with myotubes grown in standard media containing foetal calf serum with or without chicken embryonal extract, the myotubes which developed in presence of GPAG from the same muscle cell suspension were more numerous (but thinner), had a higher resting membrane potential and survived longer. The creatine kinase activity per dish showed no significant difference. The number of fibroblasts was kept low in muscle cultures grown in medium with GPAG. In combined neural and muscle cultures, the myotubes started to contract within 24 h after addition of dissociated spinal cord cells and miniature endplate potentials could be registered documenting the presence of functional neuromuscular contacts.

666 "IN VITRO" CELLULAR RESPONSE TO MODIFIED COLLAGEN-BASED MATERIAL
Sabita Srivastava(1), Laszlo Juhasz(1), Stephen Gorham(2), Archie Robertson(1).
(1) Bioproduct Development Laboratory, University of Strathclyde, Glasgow U.K., (2) Devro Limited, Glasgow, U.K.

The objective of this study is to investigate cellular and predict biological tissue response of a new range of Modified Collagen-based Material (MCM) capable of influencing cellular activities "in vivo" which can subsequently lead to enhanced tissue ingrowth and regeneration. Cell culture can be considered an extremely practical and sensitive method of assessing biocompatibility.
The basic approach of cytocompatibility is cellular interaction at the interface allowing for "in vitro" evaluation of functional performance of MCM.
This involves placing test specimens in direct contact with mouse fibroblast cells (L929) grown to confluence. The results are evaluated microscopically after 24 hrs incubation, using appropriate positive and negative controls. A cytocompatible response is characterised by viable cells exhibiting normal morphological features at the interface. It was found necessary to either vitally stain the cells or the biomaterial itself to emphasise cellular infiltration and colonisation of the biomaterial.
The versitility of this relatively simple and inexpensive cell culture method offers several advantages over "in vivo" tests which include;-
greater sensitivity, reproducibility, rapidity and conservation of test materials.

667 THE IN-VITRO DETECTION OF METABOLISM-MEDIATED TOXICITY.
S.A. Horner, J.R. Fry[1], R.H. Clothier and M. Balls. Departments of Human Morphology and [1]Physiology & Pharmacology, University of Nottingham Medical School, Nottingham NG7 2UH, UK.

Most of the cell lines used in in vitro cytotoxicity tests lack significant drug metabolising capability and cannot be used for the detection of the metabolism-mediated toxicity of chemicals (F.J. Wiebel et al., Toxicology 17, 123, 1980). In an attempt to overcome this problem, we have developed a two-component assay system, which comprises target cells of a non-metabolising cell line (V79 Chinese hamster fibroblasts) and an activating system in the form of a mammalian liver subcellular 9000xg supernatant (S9) or microsomal fraction. We have demonstrated in vitro the metabolism-mediated cytotoxicity of cyclophosphamide, which is based on the production of relatively stable reactive metabolites (S.A. Horner et al., Xenobiotica 15, 681, 1985). The Cytochrome P450-dependence of this toxicity was demonstrable using S9 fractions from the livers of differentially-induced animals, in the presence or absence of SKF525A, a Cytochrome P450 inhibitor. We then studied the cytotoxicity of bromobenzene, a chemical activated by Cytochrome P450 metabolism to a less stable metabolite. Significant cytotoxicity was only demonstrable when the assay system incorporated an S9 with P450 enzyme activity increased by phenobarbital induction and glutathione activity depleted by diethylmaleate pretreatment (S.A. Horner et al., submitted for publication). Paracetamol, which is known to be metabolised via Cytochrome P448 to a highly unstable, reactive intermediate, represents a third class of chemical, since we have to date been unable to demonstrate its cytotoxicity despite maximal P448 induction and glutathione depletion. We are currently attempting to modify the procedure so that toxicity resulting from such very short-lived metabolites can be detected. Meanwhile, it can be concluded that the degree of sophistication required in in vitro procedures for detecting metabolism-mediated toxicity depends on the stability of the metabolites produced.

668 THE EFFECT OF LIPOPROTEIN TREATMENT ON THE 3[H] THYMIDINE INCORPORATION OF CONTRACTILE TYPE BOVINE AORTIC SMOOTH MUSCLE CELLS (SMC) IN CONFLUENT CULTURE

Anna Tompa (1), Anna Kádár (2), and Magda Bihari-Varga (2), National Institute of Occupational Health, Budapest, 1450, Nagyvárad t. 2. Hungary, Semmelweis Medical University, 2nd Department of Pathology, 1450 Budapest, Hungary

The exact role of elevated low density lipoprotein (LDL) in the initiation of atherogenesis is still unknown. Confluent cultures of 3rd passage of newborn bovine aortic SMC were treated for 7 days with human LDL, very low density lipoprotein (VLDL) and high density lipoprotein (HDL) using 125, 128 and 125µg/ml concentration of these proteins respectively in D-MEM, completed with 5% fetal calf serum. At the time of treatment (12 days after seeding) the SMC culture was identified by light and electron microscopy. At this time the majority of the cells were in so called "contractile state", elongated in shape, rich in myofilaments and glycogen granules, while the cytoplasm had relatively few membranes of rEr and Golgi system. The cell to cell contacts were very intimate and developed strong adhesions of cell membranes and invaginations of microvilli. It is suggested, that the contractile state of this cell type occurs as a result of the close cell to cell interaction helped by the cell surface glycosaminoglycan content produced by confluency. The level of the labeled thymidine incorporation rate was determined by scintillation counting and compared to untreated controls. The uptake of the lipoproteins was followed by electron microscopy using colloidal gold labeling technique. Although the lipoproteins entered into the cells they did not exert any cell proliferating stimuli on the confluent monolayer. The effect of lipoproteins both on DNA synthesis and on cell proliferation of growing SMC needs further investigations.

669 LOCALIZATION OF EXOGENOUS ARACHIDONIC ACID IN CULTURED ENDOTHELIAL AND SMOOTH MUSCLE CELLS: AN AUTORADIOGRAPHIC STUDY.

Serban I.Tasca, Zorina Galis, Institute of Cellular Biology and Pathology, Bucharest - 79691, Romania

Uptake and subcellular localization of arachidonic acid (AA) in cultured endothelial cells (EC) and smooth muscle cells (SMC), isolated from bovine aorta, were investigated using autoradiography. The cells were incubated with ^{14}C-AA, and the uptake was monitored by liquid scintillation counting; some culture plates were processed in situ for electron microscopy (EM). For autoradiography, the loop technique (Caro and Van Tubergen, 1962) was applied using a L_4 Ilford nuclear emulsion. The counting of the autoradiographic silver grains was carried out by the direct allocation method. The uptake of AA had the following kinetics: half of the total radioactivity was taken up by the EC in the first 15 min and then plateaued after 90 min. The AA uptake by EC was concentration and temperature dependent but remained unaltered by indomethacin. At the EM level, the highest grain surface densities on the EC were associated with the mitochondria, lysosomes, and the Golgi apparatus. The cytoplasm (including endoplasmic reticulum, ribosomes, and small vesicles) had a high labelling intensity. The least labelled subcellular structure was the nucleus. The grain linear density on the nuclear envelope was greater than that on the plasmalemma. In SMC the quantitative estimation of the grain distribution revealed a higher density with lipid droplets and a lower one in the cytoplasm and nucleus. The linear densities of the nuclear envelope and plasmalemma were similar. Another way to asses the subcellular compartment labelling intensity was to express the individual grain count as a percentage of the total cell-associated labelling. In both studied cell types half of the grains was spread over the cytoplasm. About 20% of the grains were found in the EC nucleus and SMC lipid droplets respectively. The AA uptake by EC and SMC was demonstrated biochemically and confirmed autoradiographically. This is the first attempt to study the AA autoradiographic localization at the EM level in the EC and SMC. (Supported by Ministry of Education, Romania, and by NIH Grant HL-26343 to N. and M.Simionescu).

670 THE EFFECTS OF CORTISOL AND PTH ON ALKALINE PHOSPHATASE ACTIVITY AND DNA CONTENT IN FETAL RAT OSTEOBLAST-LIKE CELLS.

M.P.M. Herrmann-Erlee, J.M. van der Meer and G.H. Okker-Reitsma. Laboratory of Cell Biology and Histology, University of Leiden, Rijnsburgerweg 10, 2333 AA Leiden, The Netherlands.

Divergent actions of steroid hormones on bone cells have been described. The present study was designed to examine the effects of various concentrations cortisol (10nM-1µM) on isolated fetal rat osteoblast-like cells (OB-cells). OB-cells were isolated from 20-day-old fetal rat calvaria by collagenase treatment. Fibroblast-like cells, isolated by the first digestion, were discarded. OB-cells were cultured in α-MEM with 10% fetal calf serum. The medium was refreshed every third day. The OB-cells were precultured in control medium for 24 hrs, thereafter in a medium supplemented with cortisol or PTH up to 9 days. DNA content and alkaline phosphatase (AP) activity were measured as a parameter for proliferation and differentiation respectively. The results indicate that cortisol (100nM) stimulated significantly the DNA content and the AP activity already after 2 days. The stimulatory effect on DNA content increased during the following culture days. However, the stimulation of DNA content and AP activity was not observed when the OB-cells were precultured in control medium for 2-7 days. In contrast, addition of PTH (80nM) to OB-cells under similar conditions, significantly reduced the DNA content and the AP activity during the whole culture period. This relative decrease in content and activity was found with every period of preculture. This PTH effect was independent of the presence of serum in the medium. When PTH and cortisol were added together, cortisol almost completely abolished the PTH induced reduction of DNA content and AP activity.

These results indicate that the stimulation of proliferation and differentiation of rat OB-cells by cortisol is dependent on the functional condition in which the cells are stimulated. PTH, in contrast to cortisol, inhibits growth and differentiation of OB-cells and this effect is independent of the metabolic condition.

671 CHANGES IN THE PROTEIN PATTERNS OF HUMAN EUE CELLS GROWN IN HYPERTONIC MEDIUM.
M.G. Manfredi Romanini(1), A. Fraschini (1), A.M. Fuhrman Conti(2), G. Gasperi(1), C. Pellicciari(1). (1) Dip. Biologia Animale, Piazza Botta 10, I-27100 Pavia and (2) Dip. Biologia e Genetica, Via Viotti 5, I-20133 Milano, Italy.

In human epithelioid cells (EUE) grown for 4 and 10 days in either isotonic (0.137 M NaCl) or hypertonic (0.274 M NaCl) medium, the protein patterns have been studied by both biochemical and histochemical methods. Electrophoretic separation on concentration gradient (7.5-15% acrylamide gel) showed the presence, in H cultures, of at least one major protein band, which is slightly anodic compared to I controls, and exhibits a molecular weight of about 50,000. After isoelectrofocusing (pH range: 3 to 10), the same H samples showed at least two protein bands between pH 4.5 and 6, which were absent in the I controls.
Accordingly, after cytophotometric determinations on single cells of the non histone protein (NHP) content (Fast Green positive material at pH 4.5), a significant increase was observed in H cell populations compared to I controls, such a component being mainly localized in the nucleus. The apparent isoelectric point, which was determined by cytochemical methods on cells grown in H medium for 46 days, proved to be shifted from pH 4 (of the I controls) to pH 4.35.
These results may be consistent both with the evidence that the H stress affects cytoskeletal proteins (Bolognani Fantin et al., 1966) and with our previous cytochemical results suggesting a nuclear rebound of hypertonicity on EUE cells (Pellicciari et al., 1986; Manfredi Romanini et al., 1986).

672 ULTRASTRUCTURAL CHARACTERISTICS OF RAT AND MOUSE SUBSTANTIA NIGRA /SN/ IN ORGANOTYPIC CULTURES. Krystyna Renkawek, Elżbieta Kida. Institute of Medical and Clinical Research Center, Polish Academy of Sciences, Warsaw, Poland.

Substantia nigra isolated from 16-17-day- old fetuses and new-born rats and mice was cultivated in Maximow assemblies up to 12 days in vitro. The explant organization and the outgrowth rate was similar in both species studied. Ultrastructural study revealed a predominance of dark cell type in cultures from embryonic rat and mouse mesencephalon and a few synaptic contacts, whereas, the cultures from newborn mouse showed a great number of light cells, numerous synapses and processes with dense core vesicles. These differences in the cell composition and synaptic content may reflect dissimilar toxic properties of the specific neurotoxin MPTP / N-methyl-4-phenyl-1,2,3,6-hydroxypyridine / on nigrostriatal neurons in rat and mouse.

673 SYNTHESIS OF DNA, KINETICS OF ITS CONTENT IN THE CEREBELLUM CORTEX CELLS OF NEWBORN RATS IN ORGANOTYPIC CULTURE. Yu.A.Magakian, N.U.Nadzharian, N.A.Koltukhcheva. Institute of Experimental Biology of the Academy of Sciences of the Armenian SSR, 375044, Yerevan, Hasratian str.7, USSR

Synthesis and content of DNA in the cerebellar cortex cells in the process of terminal differentiation in culture were studied, this presents interest since integrative links are broken up and intercellular relations are preserved. Previously such investigations were performed only in vivo. Synthesis and amount of DNA were analysed at days 1, 12 and 32 of cultivation. At day 1 Purkinje cells (PC) and granule cells were diploid, DNA synthesis was observed in a minor fraction of glial cells. Nuclei of PC were 2-2.5 times as large as nuclei of glial cells and 3-4 times larger than those of granule cells. At day 12 all the nuclei grew in size some 1.5-fold. At this time, not more than 20% of PC were 2c, 60% were 4c and the rest of PC were at intermediate stages of DNA synthesis. 60% of glial cells were 2c the rest of them synthesized DNA. Among granule cells H2c elements were detected. At day 32 the number of 4c PC increased, the number of 2c cells was not changed, 8c cells were also found. No dividing PC observed. The number of 2c glial cells decreased (to the value of 40%), and the number of 4c cells slightly increased. Data of cytophotometry were confirmed by autoradiographic studies. PC active incorporated thymidine and at day 12 almost all of PC became labelled. Glial cells also incorporated thymidine actively and a very small number of granule cells were also labelled. Thus, in nuclei of PC in vitro replicative synthesis of DNA is induced which does not lead to division. These data indicate that differentiating neurons of CNS preserve the ability to synthesize DNA in certain conditions.

674 RADIOSENSITIVITY OF PERIPHERAL BLOOD CFUf OF TURTLE (TESTUDO HORSFIELDI) IN MONOLAYER CULTURE

A.A.Turdiev, I.G.Bogdanova-Berezovskaya Institute of Biochemistry, Uzbek Academy of Sciences, Tashkent, USSR

The efficiecy of fibroblast colony-forming(CFUf) of Central Asian turtle peripheral blood has been investigated before and after the radiation. The suspension of leukocytes has been exposed to ^{60}Co γ-rays with radiating power 4,5 R/sec at +24 - 25° at the dose 300-1500 R. The preservation of their reproduction capability has been taken as a criterion of CFUf precursors survival in case the minimum colony is made of no less than than 100 cells. The "effect-dose" curve has sigma-like shape, D_f=370 R, D_{37}=620 R, extrapolation number n=4,4. The average inactivation dose for fibroblast precursors of Central Asian turtle is $\bar{D}$=620 R, and the "minimum of adsorbtion events" is S=7. The probability of repair processes during the radiation is Ri$\leq$0,026. According to the data of A.F.Panasyuk (1973) on guinea-pigs CFUf $\bar{D}$=236 R, S=2,26, Ri$\leq$0,006. The comparison of $\bar{D}$, S, Ri, CFUf of Central Asian turtle with the corresponding parameters of mammals cells alows to suggest that the high turtle resistans to radiation alongside with the other factors revealed on the organism level is also explained by the high cell stability to ionizing radiation.

675 VARIATION OF GAS COMPOSITION IN STALKS OF SOMACLONAL PLANTS AND DOUBLED HAPLOIDS OF WINTER WHEAT. F.Sági (1), P.Bornemisza (2), L.Mózsik (1), B.Schlenk (2), M. Csatlós (2), J.Pauk (1). (1) Cereal Research Institute, H-6701 Szeged, P.O.B. 391, (2) Nuclear Research Institute, Hung. Acad. of Sci., H-4001 Debrecen, P.O.B. 51.

Internal gas composition of stalks was measured using an introduced gas sampling metal capillary and a quadrupole mass spectrometer in control wheat plants and individuals regenerated from somatic and anther calluses, respectively. The plants were grown and kept during the measurement in a growth chamber. Quantity of the gaseous components present in the stalk atmosphere was expressed in ratios to the practically constant endogenous Ar level. The stalk atmosphere contained more water vapour, 20-40 times more carbon dioxide and somewhat less oxygen than the laboratory atmosphere. The internal carbon dioxide concentration was dependent on light/darkness changes, temperature, water stress or maturation, and showed significant inter- and intravarietal variation. The stalk carbon dioxide represents probably a metabolic fraction. In contrast to the homozygote doubled haploids (DHs), erdogenous carbon dioxide did not vary more in somaclonal plants than in the controls. In a DH population, wheat plants with extremely high endogenous carbon dioxide and relatively low oxygen content were found. It is supposed that these plants possess a greater potential for phytomass production.

676 IN SITU HYBRIDIZATION WITH RADIOACTIVELY LABELED DNA PROBES: TECHNICAL PROBLEMS AND POSSIBLE APPLICATIONS:

M. Schardin*, A. Brückner*, G. Rappold**, T. Cremer*
* Institut für Anthropologie und Humangenetik, INF 328, D-6900 Heidelberg FRG
**present address: EMBL, Meyerhofstr. 2, D-6900 Heidelberg FRG

In situ hybridization with radioactively labeled probes will be described, and the effect of different hybridization conditions will be demonstrated. Hybridizations were performed on different types of human cells with various DNA probes. Repetitive sequences were labeled with one nucleotide, namely ^{3}H dTTP, while for single copy sequences the sensitivity was increased by using probes with three or four labeled nucleotides.
Applications include for example the localization of cloned DNA sequences to specific chromosomal subregions. In addition, we have used chromosome specific DNA probes to identify the number and position of the respective chromosomal domain in interphase nuclei. This approach can be used to investigate the chromosome topography in normal and aberrant cells.

677 NON-AUTORADIOGRAPHIC IN SITU HYBRIDIZATION WITH SPECIAL EMPHASIS ON THE AAF-PROCEDURE

Jim Landegent, Nico Jansen in de Wal and Rob Baan. Department of Histochemistry and Cytochemistry, Sylvius Laboratories, University of Leiden, Leiden, The Netherlands.

For nucleic acid hybridization purposes, methods have been developed in which the radioactive markers are replaced by labels such as fluorochromes and cytochemical detectable enzymes. The various procedures available today differ only in the introduction of these alternative labels at the target sequences, and can be classified as direct or indirect methods. In the former, the label is coupled directly to the hybridization probes. In the latter, the probes are rendered immunogenic by hapten modification.

A survey of the principles of several techniques, with their advantages and limitations will be presented. Special attention will be paid to an indirect method, which involves chemical modification of the nucleic acid probes (DNA or RNA) with the hapten 2-acetylaminofluorene (AAF), and hybrid detection via anti-AAF antibodies. Also various visualization procedures pertaining to optimal results will be illustrated. Our efforts, so far, have resulted in a sensitivity that allows the detection of (large) unique sequences in the mammalian genome.
(Nature 317:175-177 (1985).

678 MERCURY-LABELED NUCLEIC ACID PROBES FOR HYBRIDOCYTOCHEMISTRY

Ton Hopman, Joop Wiegant and Piet van Duijn, Dept. of Histochemistry and Cytochemistry, Leiden University, 72 Wassenaarseweg, 2333 AL Leiden, The Netherlands.

In the last few years, several non-radioactive methods have been developed for the detection of specific nucleic acid sequences in biological preparations. Different markers can be directly coupled to the nucleic acid probes or immunocytochemically introduced after the hybridization of haptenized probes. All these methods imply the introduction of the marker or hapten molecules during the in situ hybridization of the probe.

We developed a method in which the hapten is introduced after the hybridization of the probe. Nucleic acid probes can be easily modified with mercuric acetate at 50°C, during which incubation mercury atoms are covalently coupled to the C-5 position of the pyrimidine bases (U,C). This modification does not affect the hybridization efficiency of the probe and the introduced mercury possesses a high affinity for sulphydryl compounds. By this approach sulphydryl groups containing derivatives of trinitrophenol, biotin and fluorescein could be coupled to hybridized mercurated probes and detected immunocytochemically. The technique is very sensitive and enables detection of highly and middle repeated sequences, as well as the detection of single copy genes, in this case about 16 kb of the human thyroglobulin gene.

679 BASIC ASPECTS OF IN SITU HYBRIDIZATION

A.K. Raap, J.G.J. Marijnen and H. Vrolijk, Department of Histochemistry and Cytochemistry, Leiden University, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands.

The crucial step in in situ hybridization is the annealing of labeled nucleic acid sequences to complementary target sequences in morphologically preserved cells or chromosomes. For this purpose, double stranded (ds) DNA probes and ds target sequences are denatured before the in situ hybridization. Denaturation of tissues and chromosomes will lead to loss of target DNA sequences from the preparations. In addition, renaturation of probe DNA in solution and in situ renaturation of target DNA sequences may adversely influence the actual hybridization of probe and target.
Cytochemical analyses showed that, dependent on the type of denaturation, 20 to 60% of DNA is lost during denaturation. During hybridization another 20% of DNA is lost. Of the remaining DNA 20 to 40% will renature rapidly. The effect of probe renaturation in solution can, however, be benificial for in situ hybridizations with cloned DNA, since network formation by vector sequences may results in binding of large amounts of probe to the target. Alternatives for in situ hybridizations with ds DNA probes are the use of single stranded (ss) DNA or RNA probes. Especially ss RNA probes may be useful since high stringency hybridization conditions can be designed at which target ds DNA denatures.

680 T-DNA INSERTION SITES IN PLANT CHROMOSOMES AS DETERMINED BY IN SITU HYBRIDIZATION.

P.F. Ambros (1,2), M.A. Matzke (2) and A.J.M. Matzke (2). (1) St. Anna Children's Hospital, Kinderspitalgasse 6, A-1090 Vienna, Austria. (2) Institute of Molecular Biology, Austrian Academy of Sciences, Billrothstr. 11, A-5020 Salzburg, Austria.

By using non-isotopic in situ hybridization methods we were able to visualize a unique sequence - the T-DNA of Agrobacterium rhizogenes root inducing (Ri) plasmid - in plant chromosomes. The biotin/streptavidin system (Langer-Safer et al. 1982) combined with a specific microscopical set up (Landegent et al. 1985) made it possible to localize the single copy insert (approx. size of the T-DNA is 15 kb) on Crepis capillaris chromosomes. Southern blot analysis of the plant DNA probed with T-DNA fragments revealed that a single copy of T-DNA was present in the transformed root lines. A consistant hybridization signal on a single chromosome of the complement was found in all analyzed root lines. Only in one line were two non homologous chromosomes involved, indicating two separate insertion events. A comparison of T-DNA insertion sites in four different transformed Crepis root lines suggests that T-DNA integrates into random locations in the Crepis genome.

Langer-Safer, P.R., M. Levine, and D.C. Ward. PNAS 79, 4381-4385 (1982).
Landegent, J.E., N.J. in de Wal, G.-J.B. van Ommen, F. Baas, J.J.M. de Vijlder, P. van Duijn and M. van der Ploeg. Nature 317, 175-177 (1985).

681 APPLICABILITY OF HYBRIDOCYTOCHEMICAL PROCEDURES IN BIOMEDICAL RESEARCH AND CLINICAL DIAGNOSIS

M. van der Ploeg and P. van Duijn, Dept. of Histochemistry and Cytochemistry, Leiden University, 72 Wassenaarseweg, 2333 AL Leiden, The Netherlands.

Hybridocytochemistry, which allows the detection and localization of specific nucleic acid sequences in microscopic preparations, has the potential to become extremely important both in basic research and in diagnostic medicine. One of the advantages of hybridocytochemical procedures is that they allow genetic information presently gathered in pathology tissue archives to be opened to retrospective investigations as appropriate probes become available, while - most often - diagnosis at the protein level is no longer possible in such materials.
The recent development of a method to perform in situ hybridization on interphase nuclei in suspension opens new possibilities for rapid and reliable flow cytometric screening. Now that double-hybridization has become possible the detection of separate target DNAs simultaneously within one microscopic preparation is also feasible. At present the hybridization procedures using non-radioactive labeling - although still slightly less sensitive than the radioactive ones - have obtained a sensitivity that is sufficient for the detection of e.g. cytomegalovirus DNA in clinical urine and buffy coat cell samples. We successfully used the biotin- or AAF-modification for the detection of JC-virus in a case of progressive multifocal leucoencephalopathy. Several other examples of applications will be presented. In cytogenetics, better localization was obtained for the position of the Huntington's Disease linked cosmid c5,5 and also trisomy of chromosome 18 in human interphase nuclei could be easily detected.

Trask et al. Science 230:1401-1403, 1985

682 PRODUCTION OF MONOCLONAL ANTIBODIES USING AN IN VITRO IMMUNIZATION PROCEDURE.

R.P.J. Oude Elferink(1), M. de Boer(1), F.A. Ossendorp(2), G. ten Voorde(1), P. Bruning(2), J. Hilgers(2), and J.M. Tager(1). (1)Laboratory of Biochemistry, Biotechnology Centre, University of Amsterdam, P.O. Box 20151, 1000 HD Amsterdam, The Netherlands, and (2)Division of Tumor Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.

Ossendorp, de Boer and coworkers [1,2] have recently developed a procedure for the production of monoclonal antibodies using in vitro immunization in a serum-free medium. Initial experiments were carried out using human thyroglobulin as antigen. We have subsequently used this method with success for the production of monoclonal antibodies against a variety of antigens, including a synthetic peptide corresponding to a hormonogenic sequence of human thyroglobulin [3] and the lysosomal enzyme α-glucosidase. The properties of the antibodies obtained using, as antigens, precursor and mature forms of α-glucosidase isolated from human urine [4] and placenta, respectively, will be discussed.

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3. de Boer, M., de Vijlder, J.J., Ossendorp, F.A., Bruning, P.F., and Tager J.M. (1986) Mol. Immunol., submitted.
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683 RAPID MONOCLONAL ANTIBODY SCREENING BY WESTERN BLOTTING: A NEW METHOD.

Andrew E. Levin and Daniel Branton, Dept. of Cellular and Developmental Biology, Harvard University, 16 Divinity Ave., Cambridge, MA 02138, USA.

We have developed a method for efficient western blot screening of monoclonal antibodies to coated vesicle antigens which relies upon a new device termed the Miniblotter. Using this device, we were able to test 28 hybridoma culture supernatants simultaneously on a single nitrocellulose blot the size of a standard minigel. The volume of each supernatant required for the assay was 100 microliters, permitting us to sample microtiter plate wells containing hybridoma colonies at an early stage in growth. By this method, we have isolated a variety of antibodies directed against minor antigens of coated vesicles, in addition to antibodies against clathrin. Hybridomas selected in this manner secrete antibodies which recognized denatured antigens on immunoblots, a distinct advantage in characterizing the antigens. We believe that this method may prove of general interest to others screening monoclonal antibodies.

684 SPECIFITY OF MOUSE MONOCLONAL ANTIBODIES AGAINST CALMODULIN

Eva Schumacher-Wittkopf, Gabriel Rutter, Klaus Mannweiler. Heinrich-Pette-Institut für experimentelle Virologie und Immunologie, Martinistr. 52, 2000 Hamburg 20, Fed.Rep. Germany

Calmodulin, a calcium-binding ubiquitous protein is a very small molecule (16.7kd) with a highly conserved structure. Consequently it is poorly antigenic and produce normally only low or undetectable levels of serum antibodies. A positive immune response against calmodulin could be obtained in Balb/c mice with very sophisticated immunization techniques. Spleens of antibody producing mice were dissociated and fused with a non-secreting myeloma cell line (NSI 1) by application of the hybridoma technique (Köhler, Milstein Nature 256, 1975). The production of the monoclonal antibodies was determined by ELISA.
8 different monoclonal antibodies against calmodulin could be isolated. They were characterised by screening in ELISA , immunoblotting and immunofluorescence. One of these antibodies reacts very strongly with the antigen calmodulin in ELISA. It also decorates the mitotic apparatus when viewed by immunofluorescence. The other antibodies give a weak reaction with the antigen calmodulin in ELISA. Immunofluorescence studies with these antibodies in HeLa-cells demonstrate their binding at cytoskeletal components.

685 ULTRATHIN CRYOSECTIONS OF CULTURED CELLS MONOLAYERS : A NEW METHOD FOR ON-GRID LABELLING OF CYTOSKELETAL PROTEINS.

G. Langanger, J. Leunissen and J. De Mey, Dept. of Life Sciences, Janssen Pharmaceutica Research Laboratories, B-2340 Beerse, Belgium.

Ultrastructural localization of cytoskeletal proteins in cultured cells has mainly been achieved by pre-embedding labelling. To circumvent the major disadvantages of this method : detergent extraction and poor permeability of the marker into densly packed structures, we have developed a procedure that allows the ultra-thin cryosectioning of cell monolayers nearly parallel to the substrate and efficient antibody labelling of well fixed cells. The method is based on the use of polyacrylamide and N-N'-diallyltartardiamide as water soluble and reversible embedding medium. The gel gives an excellent intra- and extracellular support during cutting and is easily dissolved by Na-periodate. Sections labelled with antibodies to tubulin, actin, α-actinin, filamin and 5 or 10 nm gold probes show the staining patterns characteristic to these antibodies. The excellent preservation and sharp delineation of cellular structures, membranes as well as filaments, can be fully revealed by negative staining.

686 **IMMUNOGOLD LABELLING OF DINOFLAGELLATE LUCIFERASE AFTER FAST FREEZE FIXATION.** M.T.Nicolas(1), G.Nicolas(2), J.M.Bassot(1) and J.W.Hastings(3). (1)Laboratoire de Bioluminescence, CNRS, Gif sur Yvette. (2)Laboratoire de Technologie, CNRS, 105 Bd Raspail, 75006 Paris, France. (3)Biological Laboratories, Harvard University, Cambridge 02138 USA.

Dinoflagellates such as Pyrocystis or Gonyaulax emit a flash of 100 msec upon stimulation. The bioluminescent reaction involves a luciferin and a luciferase. It is triggered by H^+ ions resulting from an action potential. The microsources of luminescence, often also fluorescent, are clearly distinct in vivo, but their ultrastructural identity had to be ascertained. Using an antiluciferase antibody and immunogold staining(IGS) after glutaraldehyde fixation, we have first shown that,in addition to some trichocyst sheats, dense vesicles are labelled. They are always membrane bounded and situated in the cortical cytoplasm or apparently floating free in the vacuolar space.

Fast freeze fixation followed by freeze substitution (FFFS) now leads to a similar IGS localisation, but improves considerably the preservation. The previous "dense vesicles" appear to be in fact local cytoplasmic densifications devoid of surrounding membrane when they arise from the Golgi area; then they migrate, contact a vacuolar membrane and finally protrude in the vacuole as hanging drops, nearly surrounded by the vacuole membrane but still connected by a narrow neck to the cytoplasm. In such situation, these dense bodies or phanisomes can be triggered by the action potential propagated along the vacuolar membrane.

FFFS is able to catch transient cytological events; it also avoids the osmotic artifacts du to chemical fixation. The generalisation of its use in immunocytochemistry of isolated cells must be considered.

687 NEW MODES OF VISUALIZATION OF THE Ag-NOR PROTEINS AT THE OPTICAL AND AT THE ULTRASTRUCTURAL LEVELS. D. Ploton, M. Menager, J.J. Adnet. Laboratoire d'Histologie, Faculté de Médecine, Reims, France.

The argyrophilia of the Ag-NOR proteins (Argyrophilic proteins of the Nucleolar Organizer Region) is a preferential marker both of rDNA and of its actual or potential level of transcription. Thus, silver staining is a very useful tool for the study of the functionnal substructures of the nucleolus. In order to increase the precision of the technique at the optical level we found necessary to improve the conditions both of the staining and of the visualization of the Ag dots. A very specific reaction was obtained by performing the staining at 20°C on various materials : cells in smears, spread chromosomes, sections of plastic or paraffin embedded cells and tissues.

The Ag dots (0.5 µm) were visualized with bright-field, Nomarski contrast and reflected light. Compared to bright-field the use of reflected light, which is based on the ability of silver to reflect incident light specificaly, gives images with a very improved resolution. This method allowed us for the demonstration of the bipartite or tripartite structure of the metaphasic NORs and for the demonstration of very numerous small argyrophilic spheres during early telophase which fuse to form interphasic nucleoli.

Due to the absence of background the study of the repartition of the Ag-NOR proteins at the ultrastructural level was also feasible within thick sections 0.1 to 1 µm by using low to high voltage (75 to 300 kV).

The observation of stereo-pairs allowed to access to the real 3 D structure of the argyrophilic components. Thus spatial relationships of fibrillar centres with dense fibrillar component and the structure of metaphasic NORs were studied with a high resolution.

688 A METHOD FOR 3-D SHADED PERSPECTIVE DISPLAY OF CELL COMPONENTS.

W. Menhardt (1), J. Lockhausen (2), W.J. Dallas (3), U. Kristen (2). (1) Philips GmbH, Forschungslabor, Postfach 540840, D-2000 Hamburg 54, (2) Institut für Allg. Botanik, Universität Hamburg, D-2000 Hamburg 52, (3) Department of Radiology, University Medical Center and Optical Science Center, University of Arizona, Tucson, Arizona 85724, U.S.A.

An integrated environment for converting electron microscopic profile images into shaded perspective displays is described. Its application is to the display of various cell organelles: it can also be used for other image acquisition modalities. What separates this application from other, primary medical ones, is its complexity, since dictyosomes have been employed for the development of the display program. The surface profile structures of these organelles are among the most complicated being processed for scientific applications. Therefore, a high degree of interactivity had to be integrated into the three-dimensional image processing described. This encompasses scanning of electron micrographs, picture processing, semi-automatic contour following, contour editing, contour manipulation (translation, rotation), automatic topological connection, connection editing, automatic polygon tiling and display in several modalities.

689 THE UTILITY OF WHEAT-GERM AGGLUTININ-GOLD SURFACE LABELING TO ELIMINATE PLASMA MEMBRANE CONTAMINANTS FROM GOLGI-ENRICHED SUBCELLULAR FRACTIONS

Dwijendra K. Gupta and Alan M. Tartakoff, Case Western Reserve University School of Medicine, Institute of Pathology, 2085 Adelbert Road, Cleveland, Ohio 44106, U.S.A.

To document the composition of membranes of Golgi Complex and its subcompartments, a high resolution analytical subcellular fractionation procedure is essential. Available isopycnic fractionation procedures wherein postnuclear supernatants of tissue culture cells are fractionated indicate considerable overlap between rough microsomes, plasma membrane, and Golgi-derived elements.

In our studies of a rat myeloma, IR202, the total microsomal fraction prepared by a gel filtration procedure on Sepharose 2B is subjected to sucrose density gradient fractionation. A satisfactory resolution of rough microsomes from galactosyltransferase is obtained; however, the Golgi-enriched gradient regions are contaminated by smooth membranes, viz., plasma membrane-derived and endocytic vesicles. We have therefore developed a density-perturbation procedure to eliminate the plasma membrane contaminants.

A wheat-germ agglutinin colloidal gold conjugate which binds surface glycoproteins and glycolipids is used to label the intact myeloma cells at 4°C (30 min). The distribution of gold and alkaline p-nitrophenyl phosphatase (predominantly a plasma membrane marker) have been followed throughout subcellular fractionation. Binding of the gold conjugate leads to considerable sedimentation of this marker activity (45-67%) with the mitochondria. In the isopycnic microsomal subfractionation on sucrose (20-45%) in 50 mM Tris, pH 7.4, 100 mM KCL, 5 mM $MgCl_2$, the residual alkaline phosphatase activity is shifted to higher density, thus greatly increasing the purity of Golgi markers. The recovery and distribution of other marker activities (galactosyltransferase--trans Golgi, RNA--rough microsomes) compare well with those of the control and seem to be unaffected by lectin-gold binding to the plasma membrane.

690 THE PREPARATION OF NEUTROPHILS FROM HUMAN BLOOD ON ISOTONIC NYCODENZ GRADIENTS

Ford, T.C. (1) and Rickwood, D. (2). (1) University Department of Surgery, Bristol Royal Infirmary, Bristol, BS2 8HW, and (2) Department of Biology, University of Essex, Colchester, U.K.

The preparation of the neutrophil fraction from human blood presents problems in that the buoyant density of the neutrophils tends to be close to, or overlap with, that of erythrocytes. The use of discontinuous gradients results in low yield of neutrophils or high degree of contamination with erythrocytes. The method presented results in a neutrophil preparation of high yield and purity. Preformed gradients are prepared using Nycodenz Isotonic Medium (Nyegaard & Co., Oslo, Norway). The stock solution, density 1.146g/ml is diluted with isotonic NaCl solution to provide four aliquots of densities 1.11, 1.09, 1.07 and 1.05g/ml which are layered into a centrifuge tube (approx. 12 ml capacity) to form a discontinuous gradient of 8 ml total volume. The tube is sealed and laid horizontally for 45-60 min. at room temperature to allow a linear gradient to form. The leucocyte-rich supernatant is removed from a 20 ml blood sample after dextran sedimentation of the erythrocytes and the supernatant centrifuged at 200 x g for 10 min. to pellet the cells. The pellet is resuspended in 2 ml of isotonic saline, or the donor's own platelet-free cell-free plasma, and loaded onto the gradient. Centrifuge at 1500 x g for 15 min. All operations should be at room temperature. Mononuclear cells are found banded near the loading interface and the neutrophil fraction close to and just above the red cell band. The degree of separation of the two species depends upon the individual donor and careful fractionation is required to minimise red cell contamination. Nycodenz solutions are non toxic and not metabolised by cells.

691 INTRACELLULAR DISTRIBUTION OF IONS AND WATER

Thomas von Zglinicki, Martina Bimmler. Dept. of Electron Microscopy, Institute of Pathology, Charité, 1040 Berlin, Schumannstr. 20-21, GDR

Water fractions of intracellular compartments in rat heart muscle and liver cells have been estimated by X-ray microanalysis and scanning microdensitometry of electron micrographs from ultrathin frozen-dried cryosections. The results are not biased by freezing and freeze-drying. Water fractions range from 80.3±1.3 % of wet weight in the decondensed chromatin to only 45.1±1.7 % in mitochondria. Ionic concentrations per compartment water in the cytoplasm and the myofibrils are in accord with the electroneutrality rule and in osmotic equilibrium with the ECS. The nuclear membrane is free permeable for Na^+, K^+, and Cl^-. K^+ concentrations are found higher than expected for a simple Donnan distribution both in the condensed chromatin as compared to the decondensed and in the A-band as compared to the I-band. Most probably different activity coefficients and, therefore, different degree of binding of K^+ exists in different compartments. The Cl^-concentration in mitochondria has been measured far in excess to those expected from a Nernstian distribution. An active inward Cl^- transport in mitochondria has to be assumed, therefore.

692 METHODICAL REMARKS ON THE CHROMOSOME INVESTIGATIONS IN PARAMECIUM (CILIOPHORA, PROTOZOA).

Ewa Przyboś. Department of Systematic and Experimental Zoology, Polish Academy of Sciences, 31-016 Kraków, Slawkowska 17, Poland.

Analysis of micronuclear chromosomes of Paramecium is specially difficult as they are small, numerous, often aggregated, and the nuclear divisions (mitosis and meiosis) take place within the intact nuclear envelope. The method presented here allows more precise chromosome analysis than the squash method previously applied. The method consists of the analysis of serial sections of individual paramecia fixed (a modified Palade 1% osmic acid fixative) and embedded (Araldite epoxy resin) as for EM but sectioned at 0.5 um sections and examined in a light microscope after staining with toluidine blue. The diploid number of chromosomes was estimated by counting them in every section of a micronucleus and summed from its all successive sections (on average 11-15 sections). The estimated chromosome number was corrected by applying the correction formula (cf.Jurand A., Przybos E./1984/. Folia Biol. /Kraków/, 32:295-300) for multiple sectioning to avoid counting the same chromosome more than once.

The above described method was applied for the analysis of Paramecium jenningsi chromosomes in micronuclei in anaphase of mitosis in dividing paramecia as well as in micronuclei in metaphase of meiosis I in conjugating paramecia.

693 BIOCOMPATIBILITY OF BIODEGRADABLE SURGICAL IMPLANTS

Laszlo Juhasz(1), Stephen Gorham(2), Archie Robertson(1), Sabita Srivastava(1). (1) Bioproduct Development Laboratory, University of Strathclyde, Glasgow U.K., (2) Devro Limited, Glasgow, U.K.

A range of biodegradable material is under development for surgical prostheses with the object of aiding host tissue regeneration and to act as a temporary space filler in connective tissues.

The fundamental requirement for a bioactive tissue implant may be defined as:- (1) It should closely resemble the host tissue's structure and functional characteristics, (2) provide inductive and anti-thrombogenic performance, (3) to have controlled degredation rate to allow host cells to colonise and lay down viable tissues, (4) metabolise and produce essential elements for intra-cellular and extra-cellular needs.

On this basis the above research is in progress to develop bioactive soft tissue (BST), and bioactive hard tissue (BHT) implants for reparative and reconstructive surgery.

The principle constituent of the flexible BST implant is reconstituted bovine collagen (Type I) which has been complexed and chemically modified to improve biocompatability and long-term stability.

The structural components of the BHT implant is a biodegradable glass made from a calcium-sodium-phosphate system which dissolves at a predetermined rate at the tissue implant interface and releases essential inorganic ions into the extra-cellular matrix.

Cell culture and animal implant studies have been used to assess the biocompatibility of these biodegradable materials which have given favourable results.

694 RESPONSES OF THE SLIME MOLD PHYSARUM POLYCEPHALUM TO SIMULATED AND TO REAL NEAR WEIGHTLESSNESS
Ingrid Block (1), Wolfgang Briegleb (1), Karl-Ernst Wohlfarth-Bottermann (2). (1) DFVLR-Institute for Aerospace Medicine, Linder Höhe, D-5000 Köln 90 and (2) Institute for Cytology, University of Bonn, Ulrich-Haberland-Str. 61a, D-5300 Bonn 1, Federal Republic of Germany.

Simulation of weightlessness ("zero gravity" = "0 g") was achieved in our laboratory with the aid of the fast-rotating clinostat (about 50 revolutions per minute). A good candidate for testing the gravisensitivity of single cells which are not specialized for the perception of gravity is the acellular slime mold Physarum polycephalum. Its plasmodia show highly regulated contractions and dilatations of the cell envelope (ectoplasm) resulting in a regular endoplasmic shuttle streaming. The experiments were conducted in a "rotor-compressor" chamber (microchamber) enclosing between agar sheets ("sandwich") a plasmodium of about 6 mm in diameter. 0 g-simulation induces the following responses: increase in the frequency of the contractions and in the frequency of the shuttle streaming, increase in streaming velocity, and regulation phenomena guiding the described parameters back to normal values. Respiration-blockage experiments indicate the involvement of mitochondria in the perception and/or regulation.

In an experiment conducted in the Biorack aboard the space shuttle in real near weightlessness during the German D 1-Mission we were able to confirm the gravisensitivity of this slime mold. In this experiment only the contractile activity and the shuttle streaming were recorded with the aid of a photo diode and a cine camera, respectively.

695 MEMBRANE-CORTEX INTERACTIONS REVEALED IN VIVO IN HEAT-PRETREATED AMOEBA PROTEUS.
Andrzej Grębecki, Ewa Zaleska. Nencki Institute of Experimental Biology, PL-02093 Warszawa, ul. Pasteura 3, Poland.

According to the recent theory of generalized cortical contraction the membrane-associated MF layer around the whole cell periphery generates the motive force of amoeboid movement by increasing the intracellular pressure; the endoplasmic streaming and cell locomotion are promoted and controlled by local pressure drops in the frontal zones, where the MF cortex is disengaged from the plasma membrane and withdrawn to the granulo-hyaloplasm separation border. The new cortical layer is probably periodically rebuilt beneath the membrane. Such a dynamic pattern of membrane-cortex contacts was deduced from static EM pictures and immunofluorescence in fixed cells. Now, it has been revealed in vivo, and cinematographically recorded, in amoebae pretreated for 15-30 min. in 37-40°C. The cells round up and then, during chilling in the room temperature, the MF layer completely dissociates from the membrane and envelops the central core of granuloplasm, whereas a broad hyaloplasmic ring appears along the whole circumference. Numerous transhyaline strands develop radially across that ring; their dynamics is clearly associated to the contraction-relaxation events between the granuloplasmic core and the outer membrane. In the presence of various agents which increase the membrane mobility, new cytoplasmic sheets are regularly formed under the membrane, rhythmically separate from it, every 5-10 sec., and retract toward the cell centre. Every local dissociation of cortex from the membrane produces a rudimentary frontal zone and a transient initiation of movements typical for normal pseudopodial tips. The endocytosis is also easily induced in such cells, and extremely well recorded, inside the clear peripheral ring of hyaloplasm. An optically dense transhyaline strand develops between the future invagination site and the inner granuloplasm, it simultaneously pulls the membrane inwards, forming a funnel, and peels off the cortical sheet of cytoplasm around it. Also in this case, the submembraneous sheet is rebuilt within a few seconds and the endocytotic cycle may be rhythmically repeated.

696 CELL DEVELOPMENT AND INTERACTION, INVESTIGATED AND DOCUMENTED BY CINEMICROGRAPHY.
N. Hülsmann 1), Institut für Allgemeine Zoologie, Freie Universität Berlin, Königin-Luise-Str. 1-3, D-1000 Berlin 33, FRG. D.S. Shaw 2), School of Plant Biology, University College of North Wales, Bangor, UK. E. Cerdá-Olmedo 3), Departamento de Genética, Universidad de Sevilla, Apartado 1095, E-41080 Sevilla, Spain. K. Mendgen 4), Phytopathologie, Fakultät für Biologie der Universität Konstanz, Postfach 5560, D-7750 Konstanz, FRG. U. Wyss 5), U. Zunke 5), Institut für Phytopathologie der Universität Kiel, Olshausenstr. 40-60, D-2300 Kiel, FRG. T. Hard 2,3,4,5), D. Haarhaus 1), Institut für den Wissenschaftlichen Film, Göttingen, Nonnenstieg 72, D-3400 Göttingen, FRG.

The paper includes the following five films:

1) Motion, Ingestion and Reproduction in Reticulomyxa filosa (Rhizopoda). Change is shown between bidirectional protoplasmic streaming in plasmodial phase and unidirectional streaming in migrating phase. 1986, 5 min.
2) Phytophthora (Peronosporaceae) – Aggregation of Zoospores. Chemotaxis of zoospores and chemotropism of germ tubes are studied. With time lapse. 1985, 6 min. Supported by Ciba Geigy, Basel.
3) The Life of Yeast. Sexual and asexual reproduction of heterothallic and homothallic yeasts is studied, including mating switch of ascospore derivates. 1986, 13 min.
4) Infection and Host Response in Powdery Mildew of Barley. Infection process of Erysiphe graminis forma specialis hordei, reaction of susceptible host cells and hypersensitive reaction of resistent host cells are documented. 1986, 11 min. Supported by German Science Foundation Grant.
5) Heterodera schachtii (Nematoda) – Behaviour inside the Root (Rape). With the aid of video-enhancement (875 lines-system) the reaction of infected cells within the host's vascular cylinder is observable in vivo, as well as food withdrawal of the parasitic nematode. 1986, 12 min.

697 THE ADVANTAGES OF VIDEO-DISC IN EDUCATION

Dipl.-Soz. Bärbel Geiß. Institut für den Wissenschaftlichen Film, D-3400 Göttingen, Nonnenstieg 72, Federal Republic of Germany.

Laser Vision Video Disc may be considered as the ideal medium for schools, universities and post graduate training since it renders feasible whatever teachers and students may have missed and desired until now: variable forward and rewind speeds as well as the controlled single frame screening within split seconds. These, however, are only the most obvious advantages of this new technique. Its resistance against dirt, scratches or breaking are as advantageous as the time saving factors.

The Institut für den Wissenschaftlichen Film (IWF) has now produced a bilingual version (Germany and English) of its first video disc entitled CELL BIOLOGY - I. Functional Organization. This Laser Vision Video Disc has a running time of about 70 minutes.

CELL BIOLOGY - I.Functional Organization contains the following sections: Cell types, cell constituents, mitosis and cleavage, fission and cell motility.

698 **PHOSPHOTYROSINE ANTIBODIES AS PROBES FOR ONCOGENE CODED AND GROWTH FACTOR RECEPTOR KINASES**

P.M.Comoglio, L.Naldini, F.Di Renzo, D.Cirillo, G.Gaudino and S.Giordano
Dept.of Biomedical Sciences & Oncology, University of Torino Medical School

The proteins coded by class 1 oncogenes share with growth factor receptors the uncommon property of phosphorylating themselves as well as their protein substrates on tyrosine. Previous work from this laboratory has shown that antibodies against phosphotyrosine (P-Tyr) identifye the autophosphorylated form of EGF and PDGF receptors and are a useful probe to detect -at protein level- the expression of known class 1 oncogenes in experimental (i.e. retrovirus induced) tumors. P-Tyr antibodies have also been successfully employed to probe the activation of the rearranged c-abl gene in chronic myelogenous leukemia cells, where the oncogene is truncated, translocated from chromosome 9 to 22 and fused with an active gene, of unknown function, called bcr. The antibodies detected in "Western" blots the p210(bcr-abl) hybrid protein endowed with a novel -non regulated- tyrosine kinase activity.

By immunoprecipitation and blotting "tricks", P-Tyr antibodies were used to identify the substrates phosphorylated on tyrosine by PDGF and EGF receptors or by class-1 oncogene coded kinases. These included detergent insoluble cell matrix proteins of 130 and 70 Kd, and a plasma membrane associated protein of 36 Kd. The tyrosine-phosphorylated forms of these molecules were found to be barely detectable in cells stimulated by growth factors, but to accumulate in cells transformed by activated class 1 oncogene coded kinases, endowed with non-regulated enzymatic activity.

P-Tyr antibodies are presently used to screen the expression of still unknown class 1 oncogenes in human tumors via the detection of proteins phosphorylated at tyrosine. Among the panel of human malignacies so far examined, the antibodies detected P-Tyr-proteins of 100 Kd in a melanoma, of 80 Kd in a fibrosarcoma and a rhabdomiosarcoma, of 150- 100- 60 Kd in a gastric carcinoma, of 110 Kd in small cell lung carcinomas and of 165 Kd in a bladder carcinoma. The nature of some of these molecule was investigated in details and was found to be an altered form of a growth factor receptor, endowed with constitutive (i.e. non-regulated) tyrosine kinase activity.

These data support the idea that a number of malignacies are specifically associated with the expression of tyrosine kinases with non-regulated activity.

699 STRUCTURE - FUNCTION RELATIONSHIPS IN THE EGF-RECEPTOR, R. Kris, Biotechnology Research Center, Meloy Laboratories, 4 Research Court, Rockville, MD 20850, (301) 948-8044

Epidermal growth factor (EGF) is a small protein which acts as a mitogen for various cells bearing EGF-receptors. This specific interaction between EGF and its receptor eventually leads to stimulation of DNA synthesis 20-24 hours later. Subversion of the mitogenic signal through expression of a double truncated EGF-receptor seems to be involved in transformation by the oncogenic protein-v-erbB, carried by the acutely transforming avian erythroblastosis virus. The v-erbB protein lacks the EGF binding domain but has retained the protein-tyrosine kinase activity of the EGF-receptor, leading to a protein whose activity cannot be regulated by EGF. A similar protein is expressed in leukemias induced by the chronic avian leukosis virus which causes transfromation by the activation of the c-erbB/EGF-receptor gene by promotor-insertion mechanism. A synthetic peptide approach has allowed us to probe further the properties of the v-erbB protein.

One of the early events, following EGF binding is internalization of the receptor-EGF complex into cells via clathrin coated pits. The importance of certain structural domains of the EGF-receptor for proper internalization has been studied using genetically engineered mutants of the EGF-receptor which have been transfected into appropriate animal cells. Site specific mutagenesis and the sequential deletion of amino acids from the carboxy terminus of the EGF-receptor has allowed us to determine which sequences are necessary for proper EGF mediated endocytosis of the EGF-receptor.

700 The EGF-receptor kinase: Structure, evolution and properties of various receptor mutants, J. Schlessinger, Department of Molecular Biology, Research, Meloy Laboratories, 4 Research Court, Rockville, MD 20850 (301) 948-8044

The EGF-receptor is a 170 KD membrane glycoprotein which has 3 major functional domains, a large extra-cellular, glycosylated EGF-binding domain, a single hydrophobic trans-membrane region and a cytoplasmic kinase domain. The extracellular domain of EGF-receptor contains 2 cysteine rich clusters which reveal internal homology and repetition of the cysteine residues. The Drosophila EGF-receptor homolog contains 3 cysteine rich clusters. Questions concerning the mechanism of action and regulation of EGF-receptor were addressed by exploring properties and cellular effects of various EGF-receptor mutants introduced into cultured cells lines. Transient expression of intact EGF-receptor and various EGF-receptor mutants in COS-1 cells was achieved by using shuttle vector containing the SV-40 origin of replication. The same vector together with the DHFR gene were used to obtain stable cell lines expressing different amounts of EGF-receptor and its various mutants in CHO cells, which are devoid of EGF-receptor. A retroviral shuttle vector was used to express intact EGF-receptor and EGF-receptors mutants in mouse Ψ 2 and NIH-3T3 cells and for obtaining retroviruses containing sequences coding for the intact receptor and its various mutants. Initially we have generated constructs with deletions in the cytoplasmic domain of the EGF-receptor including a receptor mutant which has only 8 amino acids in the cytoplasmic domain (devoid of Thr 654). We have also introduced specific linkers into different restriction sites along the full size cDNA of EGF-receptor. Using this approach we have explored the role of various receptor domains in the regulation of receptor internalization, endocytosis and transformation.

701 Improved electron microscopic analysis of high molecular weight RNA. Application of positively charged carbon sandwich films for protein-free preparation of 18S ribosomal RNA.

Axel-M.Ladhoff, Dep.of ElectronMicroscopy,Inst.of Pathology,Humboldt-University Berlin,DDR-1040 Berlin,Schumannstr.20/21,Germ.Dem.Republic

A novel method for preparing charged carbon supporting films is described which improves the electron microscopic analysis of single strand secondary structure of RNA molecules. In the new method the amphiphilic cationic detergent benzyl-dimethylalkylammonium chloride (BAC) and the cationic intercalative dye ethidium bromide (EtBr) are arrested between two thin carbon layers forming a permanent charged carbon sandwich film. This supporting film is able to bind polyanionic nucleic nucleic acids via cationic "bridges" which resist the washing, staining and drying procedure. Secondary structure images of 18S ribosomal RNA from rabbit reticulocytes and rat liver prepared by this "ion-bridge-technique" are compared with known model structures gained by computer analysis.

702 STUDIES ON THE CONSTITUTIVE CELLS OF VISUAL ORGAN IN XIAMEN AMPHIOXUS I. COMPARED THE PHOTORECEPTORAL STRUCTURE WITH THE DIFFERENT BODY SECTION, ILLUMINATION AND SIZE.

Wang Deyao (Ouang Teyio), Huang Chunfa. Institute of Cell Biology, Xiamen University, Xiamen, Fujian, P.R.C.

After investigating the ultrastructure of the visual organic constitutive cell in the amphioxus (Branchiostoma belcheri Gray)+, we studied again the ultrastructure of the photoreceptors which existed in "eyesport" and other position of the nervous tube, dark adaptation (20 days) and light adaptation (12 hrs) and small (< 3cm) and big (> 5 cm) individuals. The initial results are as follows:

It is identity that is the ultrastructure of the photoreceptor between "eyesport" ant other position of the nervous tube. The microvilli in dark adaptation are longer, denser and orderer; the cristae of mitochondria are fewer and soma is bigger than those in light adaptation. In dark adaptation, the pigment particles in pigment cell are covered the photoreceptor in the form of this layer. There are many vesicles above the nucleus. After light adaptation, the pigment particles concentrate the "cup-base" from the "cup-edge". The microvilli of small individuals arrange denser, the cristae of mitochondria are fewer, above the nucleus there are abundant vesicles. In big individuals, the microvilli get thick, the big vacuole appears above the nucleus. It may be transformed by the vesicles. These changes are similar to that of the various illumination, it is relative to act both big and small individuals. The other cell organelles are very few.

Our results suggest that there is photostructure in "amphioxus eyesport", it should be able to photoresponse. The changes caused the various illumination illustrate that the illumination can cause the darange and decompose of microvilli and facilitate mitochondrial vacuolizition. It is identified with the phenomenon caused amphioxus death by illumination.

+Wang Deyao, Zheng Limou 1984, Abstract of the papers presented at the Third International Congress on Cell Biology, p277, Tokyo.

703 PLANT REGENERATION FROM GRAPEVINE CALLUS.

Pavel G. Kovalenko, Boris A. Levenko. Institute of Molecular Biology and Genetics Academy Sciences of the Ukrainian S.S.R., Kiev-252143, USSR.

Plant regeneration from callus is a necessary step to different manipulations with tissue and cells in vitro. Callus was derivated f rom young stems and leaves, using modified Murashige-Skoog medium with 2mg/l NOA, 0.2mg/l BAP, 0.01mg/l IBA, sucrose 3%, activated charcoal 0.05% and 0.7% agar "DIFCO". Primary callus was subcultured during 4 months, than it was transfered in hormones free medium for cool treatment with 4 C for three weeks. Shoots formation was obtained in modified MS medium with addition of 250mg/l inositol, sucrose 3%, ascorbic acid 30mg/l, and glucose, fructose and galactse each of 1g/l, and supplement with 3mg/l BAP, 0.34mg/l GA3, 0.7% agar "DIFCO". Morphogenic callus was subcultured in low light intensity. After shoots formation, plant regenerants were transfered on modified MS medium for rooting with addition of 2% activated charcoal, and 0.1mg/l IBA. Regenerants were multiplied in liquid medium on paper bridges. Nutrient medium was modified MS, contained 0.1mg/l IBA, or without hormones. Plants rooted under such conditions assimilated well being transfered into soil and perlit mixture. Organogenesis was obtained in following grapevines varieties: "Cabernet Sauvignon", "Antey", "Podarok Magaracha".

704 VOLUME REGULATORY BEHAVIOUR OF HUMAN BLOOD PLATELETS.

Avinoam Livne, Sergio Grinstein, Esther Mack, Aser Rothstein. Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada.

Platelets exposed to hypotonic medium undergo an initial swelling followed by a rapid shrinking (Regulatory Volume Decrease or RVD). If the RVD is blocked, the degree of swelling is in accord with osmotic behaviour. Swelling without lysis can be extensive, up to four-fold increase in volume, with complete reversibility and retention of functions such as aggregation or serotonin secretion. The rate of RVD in NaCl is rapid and is accelerated by removal of Na^+ and Cl^- from the medium (Methylglucamine sulfate > Na_2SO_4 > NaCl). It is unaffected by substitution of NO_3^- for Cl^- or by addition of bumetamide, an inhibitor of the cation-anion cotransport system. The RVD is driven largely by the KCl gradient operating through independent volume-sensitive K^+ and Cl^- pathways. Inhibition of either pathway blocks RVD. If the net KCl gradient is inward, the intial osmotic swelling is followed by secondary swelling rather than RVD. In NaCl medium, the RVD is incomplete apparently due to activation of Na^+/H^+ exchange. The uptake of Na^+ via this exchange system (and presumably of Cl^- via Cl/HCO_3 exchange) accounts for the diminished shrinking, because inhibition of the Na^+/H^+ exchange by amiloride (or by N5-N-substituted analogs) or removal of Na^+ from the medium allows complete RVD to occur. (This study was supported by the Medical Research Council of Canada, Grant # MT4665).

705 Na^+/H^+ EXCHANGE IN CULTURED RAT GLIAL CELLS ELICITED BY SHORT TERM EXPOSURE TO ASTROGLIAL GROWTH FACTOR. L. Latzkovits/1/, Csilla Torday/1/, Brigitte Pettmann/2/, G. Labourdette/2/, M. Weibel/2/, Monique Sensenbrenner/2/. /1/ Institute of Experimental Surgery, Medical School of Szeged, H-6701 Szeged, POB 464, Hungary, and /2/ Centre de Neurochimie, CNRS, 67084 Strasbourg, 5 rue Blaise Pascal, France.

Recently, Monique Sensenbrenner's group in Strasbourg has extensively purified an astroglial growth factor /AGF/ from bovine brain and has shown to stimulate the proliferation and maturation of astroblasts in primary culture. At the same time, it has been suggested by several reports from other laboratories that some growth factor effects on cell multiplication and differentiation are mediated by an amiloride sensitive membrane mechanism that exchanges external Na^+ for internal H^+. Our present work was undertaken to see whether might this Na^+/H^+ exchange system play a role in the development of AGF effects on cultured glial cells. Primary cultures of astroglial cells were derived by mechanical dissociation of newborn rat cerebral hemispheres and by subsequent cultivation of dissociated cells first for 5 days in a 10% fetal calf serum containing medium and afterwards for further 6 days in a serum-free defined Waymouth's medium as it has been described by M. Weibel et al. /1984, 1985/. The astroglial cultures obtained this way were exposed to AGF for 1-60 mins. After changing the medium for a Tyrode balanced salt solution containing either 22-Na or 86-Rb as tracer, the uptake rates of these cations were determined. It was observed that an exposure of astroglial cells to AGF for 10 mins. or for a longer period significantly increased both 22-Na and 86-Rb uptake rates. Both effects could be inhibited by amiloride. The presence of ouabain also inhibited the rise of 86-Rb influx elicited by AGF while that of 22-Na influx was not affected by ouabain.

706 ULTRASTRUCTURE OF IMMOBILIZED CELLS OF Claviceps fusiformis AND ITS BIOLOGICAL ACTIVITY ON A LONGTERM SEMICONTINUOUS CULTIVATION.

J. Ludvík, V. Křen, Z. Řeháček, O. Kofroňová, J. Kozová. Institute of Microbiology, Czechoslovak Academy of Sciences, Vídeňská 1083, 142 20 Praha 4, Czechoslovakia.

Vegetative mycelium of Claviceps fusiformis immobilized in 4 % Ca-alginate maintained in a 500-d semicontinuous cultivation produced 1200 mg/mL of clavine alkaloids on the average. Selected enzymes of metabolic pathways, i.e. glycolytic pathway, pentose cycle and TCA-cycle were assayed. Mycelial growth decreases towards the middle of the beads, due to a limited diffusion of nutrients. After 60 days the cells differentiated into the sclerotial forms exhibiting the maximal biosynthetic capacity. Cavities in the beads filled with partially lyzed cells were formed later (after 130 days). However, most cells of the biocatalyst were still viable even after 500 days. From the ultrastructural point of view, the cytoplasm of the cells became condensed after 3 days, polysaccharides disappeared and lipid synthesis centres in the cytoplasm were formed. After 60 days the cells harboured great numbers of lipid particles, mitochondria diminished and their cristae disappeared indicating a decreased respiratory capacity. After 350 - 500 days the volume of most cells enlarged many times and the cells were filled completely with large oval bodies of electrondense material.

707 PROSOMES FORM SUB-NETWORKS OF INTERMEDIARY FILAMENTS IN FUNCTION OF CELL DIFFERENTIATION.

Klaus SCHERRER (1), Maria-Fatima Grossi de Sa(1), Francis Harper(2), Jayanta K. Pal, Pierre Gounon(3), Cezar Martins de Sa(1), Omar Akhayat(1). (1) Institut JACQUES MONOD, 2 Place Jussieu - 75251 PARIS Cedex 05 (France); (2) I.R.S.C., BP n°8 - 94800 VILLEJUIF (France), (3) Centre de Cytologie Experimentale, 67, rue Maurice Gunsbourg - 94200 IVRY-SUR-SEINE (France)

Prosomes were discovered as subcomplexes of repressed messenger ribonucleoproteins (mRNP) and characterised as a specific entity of their own by electron microscopy and biochemical criteria (SCHMID et al, (1983) EMBO. J. 3 , 29; MARTINS de SA et al, (1986) J. Mol. Biol. 187 (4), 000; cf. GROSSI de SA et al, (1986) this abstract volume). Composed to 85 % of protein and 15 % of small RNA (50-150 n) the individual particle is raspberry shaped with a diameter of 12 nm. There are about 25 peptide constituants of MW 19.000 to 50.000 and pI 4-7, showing a characteristic two-dimensional electrophoretic pattern. The individual particle (600.000 MW) may contain multiple units of a single peptide-component. - Monoclonal antibodies (MABs) constructed against duck erythroblast prosomes allow to establish specific phylogenetic relationships; some cross-react with Drosophila and plant prosomes. Prosomes are antigenically and structurally related to low MW heat-shock proteins (hsp). - Dual label immunofluorescence on avian and human cells revealed that prosomes are localized on the intermediary filaments forming networks superimposable extensively onto the cytokeratin, and partially onto the vimentin and actin networks. Prosomes are also in the nucleus associated with pre-mRNA, the same MABs also label the nuclear matrix and lampbrush chromosome loops. In embryonic tissue, individual prosomal antigens are located in specific cells and developmental domains, in the nucleus and/ or (sectors of the) cytoplasm, in configurations varying with the type and stage of differentiation. It is proposed that specific prosomes accompany specific pre-mRNA and mRNA in a tissue- and differentiation-specific pattern from synthesis to expression, and that these carriers of information travel on the nuclear matrix and intermediary filaments.

708 NEUROHYPOPHYSIS OF RATS NEONATALLY TREATED WITH ESTROGEN. Vladimir Pantić, Marija Simic. Department of Histology and Embriology, Faculty of Veterinary Medicine, 11000 Beograd, Bulevar JNA 18, Yugoslavia.

The neurohypophysis of rats neonatally treated with a single or repeated doses of estrogen is the subject of this presentation. Animals treated at the age of 3 days sacrified during various stages of development up to sexual maturity. The neurohypophysis fixed in 4% glutaraldehyde, postfixed in 1% O_8O_4 and embedded in araldite was examined using a Siemens electron microscope - Elmiscop 101. The purpose of these investigations was to follow the pathway of the characteristic properties and reactions in pituicites and nerve terminals during the development of the neurohypophysis in rats neonatally treated with estrogen, and to compare them with data observed in this lobe of corresponding intact animals. Data obtained so far show thatas the result of long term effect of estrogen, the amount of neurosecretory granules in the nerve terminals of treated animals was decreased. Besides Herring bodies which were mostly dilated, membranous bodies and large lipid droplets were present in their axoplasm as well. The cytoplasm of pituicytes of animals treated with estrogen contained well developed Golgi complex, GER and large lipid droplets. These changes, including nerve terminals and pituicytes were more clearly pronounced if the animals were treated with repeated doses of estrogen.

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2. De Duve, C. (1959) Lysosomes, a new group of cytoplasmic particles. In: Hayashi, T. (ed.) *Subcellular Particles.* Ronald Press, New York.
3. Umbreit, W. E., Burris, R. H., Stauffer, I. F. (1957) *Manometric Techniques.* Burgess Publishing Co., Minneapolis.

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FORUM, Vojvode Mišića 1, *21000 Novi Sad*

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