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ABSTRACTS

12th MEETING OF THE

HUNGARIAN BIOPHYSICAL SOCIETY

24 - 26 August, 1983

BUDAPEST

INTRODUCTION

Biophysics - similarly to biochemistry and molecular genetics - plays a constantly increasing role in the development of the whole biology and it is one of the most significant promoters in the "biological revolution".

Biophysics has a well organized society in Hungary since 1960. It is not widely known that the density of the organized biophysicists in Hungary is the largest one over the world: 40 biophysicists/million of population. This fact means that the organizers of the Hungarian biology recognized very early the decisive character of biophysics. The existence of 5 biophysical departments at the Hungarian universities is another proof of this idea.

The national meeting of the Hungarian biophysicists is a regular biannual conference in Hungary. Now, the abstracts of the XIIth Meeting are presented here and they give a more or less informative picture of the scientific achievements of the biophysical research in this country. Because of the interdisciplinary nature of the studies there are lots of other results published at related meetings. Nevertheless, the main lines of the research conducted in Hungarian biophysics and the initiatives of the young scientists can be clearly seen from the abstracts.

The greatest advantage of publishing the abstracts lies in their quick informational character.

Prof. J. Tigyi
Member of the Hungarian Academy of Sciences
President of the Hungarian Biophysical Society

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BIOLOGICAL MACROMOLECULAR SYSTEMS - LIQUID CRYSTALS

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The main building units of biological macromolecular systems are the proteins, the nucleic acids and lipids. All of these molecules play an important role as structural and functional elements in the living cells. Recently special attention has been given to the liquid-crystalline properties of lipid/lipoprotein/-containing cell membranes /G.H. Brown, J.J. Wolken: Liquid Crystals and Biological Structures, Acad. Press, London, 1979./ and on the basis of these properties some problems of membrane structure and function were interpreted successfully /I.P. Sugár: Biophys. Chem. 15, 131-134/1982/. - The liquid crystalline behaviour of certain proteins and synthetic polypeptides /K. Czarniecka, E.T. Samulski, Mol. Cryst. Liqu. Cryst. 63, 205-214 /1981/ was studied in detail too, but such type of behaviour in the case of nucleic acids /nucleo-proteins/ is relatively less known. Earlier it was found that in certain bacterial viruses e.g. in phages T7 their nucleic acid interacting with the proteins has a special structural order and the ordering of the biopolymer is similar to a liquid crystalline structure /A. Fekete et al. Biophys. Struct. Mech. 9, 1-9. 1982/. - The presence of the intraphage DNA structure allows a possible interpretation of the difference in the quality and quantity of UV damage as well as in the binding of the trimethoxypsoralen to the intraphage DNA and to DNA in solution found in our laboratory /A. Fekete, Gy. Rontó: Studia biophys. 80, 165-171. 1980., J. Fidy, K. Tóth, Gy. Rontó: Studia biophys. 94, 37-39. 1983/.

ROTATIONAL DYNAMICS OF CONTRACTILE PROTEINS IN SUPRAMOLECULAR COMPLEXES

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Structural changes and dynamical phenomena in muscle and in contractile protein systems can be monitored by paramagnetic probe molecules attached to Cys-373 in actin or to the SH-2 sulfhydryl site in myosin head portion recording the changes in the anisotropic distribution of probe molecules by conventional EPR technique or following the motional rates of the rigidly attached spin labels in the saturation transfer EPR time domain /Belágyi et al. Studia Biophys. 77. 77-83 1979; Gróf et al. Symp. Nitroxide Radicals, 1979/.

We have examined the rotational properties of actin and the interaction of actin with heavy meromyosin /HMM/ in solution and in oriented filament system. HMM induces actin polymerization even at high ionic strength, the interaction of HMM with actin reduces the axial order of probe molecules and has a cooperative character. The comparison of the EPR data in oriented spin-labelled actin-HMM system and in spin-labelled glycerinated muscle fibres in rigor and during contraction leads to the conclusion that the force-generation unit of the striated muscle comprises the myosin head and a domain of the thin filament, and that the rotational motion of the domain may cause the shortening of the muscle.

NEW RESULTS IN THE INVESTIGATION OF MEMBRANE DYNAMICS

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A brief summary is given on the techniques applied for the study of dynamic characteristics of the membranes. Basic features of the methods yielding data on the distribution, lateral and rotational mobility of integral protein and glycoprotein components of the cytoplasmic membrane /fluorescence energy transfer measurements on cell surfaces, fluorescence recovery after photobleaching, phosphorescence anisotropy measurements, linear dichroism measurements and fluorescence depletion techniques/ are discussed. Sensitivity and applicability of methods yielding information of the same type are compared.

To illustrate the capability of the methods described a number of experimental systems were chosen and some new results are reported obtained using a particular type of these techniques or a combination of them. The results of the authors are mentioned as well as results of other groups.

ABOUT THE SAFETY OF ULTRASOUND

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Many of the ultrasound specialists will perhaps find it unnecessary to speak about the presumed dangers of ultrasound. We are working day after day with our medical ultrasound equipments and are entirely convinced of our and our patients' safety. However, investigations on this field are not yet finished at all. We have to know the hazard levels in the industrial application of ultrasound as well as in the medicine where judgement to the balance of risk and benefit will need to be made by the physician responsible for his patient.

ROLE OF BODY-FOREIGN SUBSTANCES IN THE FEEDING OF ANIMALS

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The per capita consumption of meat, milk and eggs is the most reliable measure of the food requirement of the population. The traditional methods of animal breeding and feeding are, however, no more suitable to meet the food requirement of the growing population.

Through feeding, animals are taking more and more body-foreign, chiefly chemical substances.

Due to the soiling of environment plants serving the purposes of animal feeding contain more and more contamination. Also different fungi of plants and their toxins are significant sources of contamination.

The by-products of food industry are used to a continuously increasing extent as animal nutrients. Considerable part of these originates from the residue after extraction of oil seeds. Thus drugs used during the extraction and toxins of plant fungi contaminate the aliment.

Beside the preservation of purity of products used for feeding, the increase of the efficiency of industrial seed mixtures made of these products is also an important task. This can be achieved by addition of so-called organ-forming materials, mineral substances, Ca, P, Mg, NaCl, trace elements, furthermore preventive materials and stimulators, synthetic vitamins and flavour substances.

The conservation of industrial mixtures is assured by anti-oxidants.

Before a week of slaughtering it is not allowed to fodder nutriments containing body-foreign substances.

Considering that approximately 50% of the food production in Hungary is exported it is imperative for us to respect not only the local sanitary regulations, but the rules concerning the food production of the COMECON, the Common Market and the USA.

On the basis of a close cooperation of veterinary and health experts these demands can be effectively satisfied.

HYDROGENASE: A KEY ENZYME IN BIOLOGICAL SOLAR ENERGY CONVERSION SYSTEMS

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Hydrogenase is an enzyme /E C class 1.12/ which catalyzes the reversible reaction $2H^+ + 2e^- \rightleftharpoons H_2$. It has been found only in prokaryotes, its physiological role is not clear. The enzyme is usually linked to the electron transport chain.

A common property of the hydrogenases purified from various bacteria is that they contain one or more iron-sulfur cluster/s/. Changes in the redox states of this iron-sulfur cluster correlate with the enzymatic activity of the protein. Hydrogenase produces molecular hydrogen in biological solar energy conversion systems when supplied with electrons of suitable redox potential. Various systems can utilize biomass or water as an electron source. The efficiency of these processes is rather low mainly because of the limited lifetime and instability of the components. Hydrogenases with remarkably high stability have been obtained from photosynthetic bacteria. Their characterization as well as an understanding of the molecular mechanism of catalytic activity and of the factors affecting specific activity need further extensive research. The results show that hydrogenase is located in the membrane of these bacteria and the hydrophobic environment plays an important role in the regulation of the enzyme function.

THE ROLE OF PLASMIDS IN RADIORESISTANCE AND MUTAGENESIS

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As known extrachromosomal genetic elements, plasmids are responsible for the antibiotic resistance of bacteria isolated in clinics. The best known called R-factors are those of the Gram negative bacteria. Plasmids can convey resistance also to different metallic ions. A wide variety of plasmids have been shown to increase the survival of bacteria exposed to UV light, ionising radiation and many chemical agents /methyl-methane-sulphonate, nitrofurantion, bleomycin, etc./. Hence, the plasmids confer resistance to a wide range of physical and chemical DNA-damaging agents. The ability of some plasmids to enhance mutagenesis following DNA damage has led to the incorporation of plasmid pk M 101, a derivate of R46, into the strains of Salmonella typhi murium used in the Ames mutagenicity test. The plasmid greatly increases the sensitivity of this test, and a number of known carcinogens that fail to induce mutations in plasmidless cells are mutagenic in the corresponding plasmid-carrying strains.

The biochemical basis of plasmid-mediated UV and ionising irradiation-protection and mutagenesis is unclear. The authors will discuss the possible mechanisms of the above effects of plasmids in connection with recent data in the literature and their own experimental results.

THE PLASMA MEMBRANE AS RADIOSENSITIVE TARGET

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Recent information on the structure and function of cellular membrane system gave new impetus for the relevant radiobiological research. The obvious final aim of all these studies is to reveal how far and by what mechanisms might the membrane-bound cellular processes be influenced by ionizing radiations, i.e. whether the membrane system is a radiation-sensitive target; further, how do the radiation-altered membranes contribute to the development of other cellular radiation effects. Data from the literature and from our investigations suggest that both the function and structure of plasma membranes react sensitively to radiation doses in the non-lethal and sub-lethal ranges. It is emphasized that more information is needed to answer the questions raised concerning the role of various energy deposition events in temporarily appearing and rapidly regenerating radiation-induced perturbation of membranes, and the possible consequences of these in the fate of cells, in the development of stochastic and non-stochastic radiation effects.

PROBING DOMAIN-DOMAIN INTERACTIONS IN ANTIBODY MOLECULES BY USING SPIN-LABELLED HAPTEN

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At molecular level the immune response is mediated through antibodies. The multiple functions of antigen recognition and the secondary effector processes are associated with discrete domains within the antibody molecule. The mechanism, by which the initial antigen recognition on the Fab part of the molecule is communicated to the distant /7 nm/ secondary binding sites on the Fc part, is still a matter of controversy. We used spin-probes to detect interactions and information transfer between the Fab and Fc parts of rabbit IgG antibody molecule.

Anti-dinitrophenyl-IgG was produced in rabbits by injection of dinitrophenol groups /DNP/ conjugated to human serum albumin. The specific anti-DNP-antibody was prepared by affinity chromatography using DNP-conjugated human IgG bound to Sepharose matrix. 2,4-Dinitrophenylhydrazone of 1-oxyl-4-oxo-2,2,6,6-tetramethylpiperidine was used as a spin-labelled hapten.

Staphylococcal protein A /SpA/, a cell wall protein, is constructed of four different structural domains. Each domain is capable of specific binding to the Fc part of immunoglobulins. This protein can be used to crosslink four IgG molecules, and monovalent proteolytic fragments may serve as

specific affinity labels of the Fc part of antibodies. In our system the spin-labelled haptens located on the Fab part of rabbit IgG were used to detect the effect of cross-linking and monitor changes in the conformational dinamics around the antigen binding site upon attachment of monovalent fragment of SpA to the distant Fc part of the molecule.

The ESR spectra reflect immobilization of the spin-labelled hapten bound to the antigen binding site of IgG molecule. The spectra can be resolved into two main components according to their mode of immobilization. The effect of SpA binding and crosslinking is interpreted in terms of signal transfer between the longitudinal domains of IgG, by the mechanism of "modulation" of the coupled conformational fluctuations of the protein matrix.

EFFECT OF TRYPSIN BINDING ON THE DYNAMIC PROPERTIES OF THE TRYPSIN INHIBITOR PROTEIN: AN NMR STUDY OF ISOTOPE EXCHANGE OF SINGLE AMIDE HYDROGENS

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Basic pancreatic trypsin inhibitor /BPTI/ has a hydrophobic core containing buried amide hydrogens. The time course of the exchange of these hydrogens can be followed individually by NMR spectroscopy. In this work the effect of trypsin-BPTI complex formation on the H-D exchange kinetics of single amide hydrogens of BPTI was studied.

Since we intended to get information on the dynamic properties of the native structure rather than on the native--unfolded equilibrium, the exchange kinetics were determined under conditions where the complex is stable and the exchange rates are proportional to the OH^- ion concentration catalyzing the chemical step of the exchange /pH 9-10; 25-40^{o}C/. Under these conditions the activation energies of H-exchange fall in the range of 10-30 kcal/mol both in the case of free and complexed BPTI. These activation energies are significantly smaller than those corresponding to a native-unfolded transition /60-80 kcal/ kcal/mol/.

It was found that trypsin binding, which does not alter the time average structure of BPTI, has significant effect on the H-exchange kinetics of the hydrophobic core. The rate constant of amide hydrogen exchange of amino acid located in

the core closest to the contact surface in the complex, Tyr 35, is decreased by more than three orders of magnitude due to complex formation. Moving away from the contact surface the influence of trypsin binding on the rate constants decreases subsequently. For amide hydrogen of amino acid located farest from the contact surface, Gln 31, the decrease of rate constant is only twofold.

EFFECT OF MECHANICAL, ELECTRICAL AND OSMOTIC PRESSURE ON THE PHASE TRANSITION PROPERTIES AND STABILITY OF PHOSPHOLIPID BILAYERS: LANDAU'S THEORY OF ONE-COMPONENT SYSTEMS

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A theoretical model has been developed to describe not only the temperature-induced phase transition of phospholipid bilayers but also the phase transitions induced by different types of pressure. The calculated pressure-temperature phase diagrams show that the first-order gel-liquid crystalline phase transition becomes a second-order one for dipalmitoylphosphatidylcholine /DPPC/ bilayers at the following critical parameters: $T_c = 38.8^oC$, $\mathcal{P}_c = -5\ 10^{-13}$ N/chain /transmembrane compression/ or $T_c = 38.2^oC$, $\mathcal{G}_c = 3\ 10^{-3}$ N/m chain /lateral tension/. The boundary of liquid crystalline and isotropic fluid phase and the limit of membrane stability are shown on the phase diagrams too. From the limit of membrane stability the calculated pore edge energy is about $4 \cdot 10^{-12}$ N. By means of the transmembrane pressure - temperature phase diagram, the diagram of transmembrane potential - temperature is calculated too. According to this latter diagram the reversible electrical breakdown phenomenon is connected with a sudden change of the membrane structure from liquid crystalline phase into fluid phase and vice versa. It is shown that the transition into the fluid phase results in the formation of hydrophilic pores and, as a consequence, a drastic increase of membrane conductivity. These pores are distributed

homogeneously at the surface of the flat membrane and their size is independent of the transmembrane potential. But at a given transmembrane potential we reach the limit of membrane stability and a complete mechanical breakdown of the flat membrane sets in. However, concerning the spherical vesicles, this critical transmembrane potential results only in local instabilities or local mechanical breakdown, i.e. formation of holes at the 'pole caps' where the largest values of the field strength vector exist. The size of these holes depends on the transmembrane potential and after removing the field a resealing process of vesicles takes place.

AB INITIO CONFORMATIONAL ANALYSIS OF MODEL PEPTIDES

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The theoretical investigation of model peptides is of great importance in understanding polypeptide and protein dynamics. Formamide and N-methylacetamide have been selected as model systems to study the nonplanarity of the peptide bond and the rotational structure of the peptide unit. Quantum chemical calculations have been performed on different conformational states of these compounds. In the calculations the pseudopotential-FSGO fragment method has been employed. It has been already proven that this method is especially well suited for the prediction of conformational properties of large molecular systems. The results are compared with experimental observations and with the conclusions of semiempirical and other ab initio investigations on model peptides.

INTERMEDIATE AND ZIGZAG /ELECTRONIC/ STATES /IS AND ZZS/ AND QUANTUM SIZE EFFECTS /QSE/ IN FINITE CRYSTALS AND BIOPOLYMERS: PROPOSALS FOR EXPERIMENTS

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First the theory of the ISs, ZZSs and QSEs are reviewed in light of data in the literature /Ahlqvist, 1982; Biczó, 1983; Cottey, 1972, 1975/. Next, it is shown that QSE experimetns /18th and 20th refs. of Ahlqvist, 1982; Jaklevic, Lambre, 1972; Lutskil, 1970 and references therein/ can give information on the ISs, because they belong to the observed class of the one--electronic QSE states. Their connections with certain problems of monolayer organizates /Möbius, 1982/ and molecular biology /Biczó, 1983; Larsson, 1982 and references therein/ are mentioned. Finally, to observe ISs, d.c. and a.c. /resonance/ tunnelling experiments of the type used by Jaklevic, Lambre, 1972 and Lutskil, 1970 as well as surface-sensitive optical absorption and photoelectron yield measurements /18th and 20th refs. of Ahlqvist, 1982/ are proposed. The former experiment would utilize the turning over /state pump/ process of the ISs /Biczó, Lukovits, 1979/, while the latter two the high instabillity of the ISs as compared to the bulk-like QSE one-electronic states.

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CELL SURFACE RETROVIRUS-RELATED ANTIGEN ANALYZED BY FLOW-CYTOMETRY

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Expression of cell membrane antigens recognized by antiserum raised against purified Friend murine leukemia virus /Fr-MuLV/ envelope glycoprotein /gp71/ /Virology, 1979, 93:159/ and by xenotropic-MuLV-coded Cell Surface Antigen /XenCSA/ specific antibodies /J. Immunol. 1979, 122:443/ was studied in the course of the development of Rauscher erythroleukemia in the spleen of Balb/c mice. Immunofluorescence techniques including a sensitive three-step sandwich procedure, were used in a simple protocol, for the simultaneous analysis of antigen exhibition and DNA content. At early stages of the disease /4-5 days after infection/ the gp71 and XenCSA-related antigen expression is enhanced mainly on S-G2/M-phase cells as compared to the majority of G1-phase cells or to the endogeneous background of uninfected cells. Later /around 10 days after infection/ an approximately tenfold increased gp71-related antigen density is reached in every phase of the cell cycle. These data suggest that the virus-induced transition from resting to proliferating state is coupled to enhanced expression of both helper and defective viral env-gene products in the cell-membrane.

ACTIVE SITE DYNAMICS IN GLYCOGEN PHOSPHORYLASE B AND ITS POSSIBLE FUNCTIONAL SIGNIFICANCE

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Pyridoxal-5-phosphate coenzyme plays an active role in the catalytic function of α-glucan phosphorylases; however, its contribution to the catalytic events is far from being understood. Being a molecule of chromophoric nature it is a useful reporter group in the broad scale of spectroscopic investigations on conformational transitions of the phosphorylase b.

Present investigations on the quenching of its fluorescence by anionic quenchers /iodide, iodate, etc./ and additional fluorescence polarization measurements refer to a quenching process of collisional /dynamic/ character taking place preferentially by bound quencher species. This hypothesis is reinforced by comparative studies with a model compound of the coenzyme /pyridoxamine-5-phosphate/ and analysis of the temperature dependence of the quenching. The binding of quenchers to the enzyme has been demonstrated by enzyme-kinetic studies. By these results the site of the quencher's binding is supposed to be the one to which the anionic substrates of the enzyme /P_i and glucose-1-phosphate/ are bound, since both quenchers have proved to be competitive inhibitors with both of the substrates. The possible functional significance of the dynamic interaction between the cofactor- and the substrate-binding subsites /on the ns time scale/ is discussed in the light of the more recent models of the catalytic mechanism of glycogen phosphorylases.

INVESTIGATION OF FLUORESCENCE RESONANCE ENERGY TRANSFER PROCESSES ON THE CELL SURFACE

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Topographical distributions of the mouse histocompatibility antigen H-2K^k and Con A binding sites were studied by fluorescence resonance energy transfer measurements performed on a cell-by-cell basis using a computer-controlled flow-cytometer capable of dual wavelengths excitations. Different monoclonal antibodies directed against H-2K^k antigens and the individual Con A molecules were labelled with FITC and TRITC and used to determine the proximity of the individual H-2K^k antigens and the individual Con A binding sites as well as the average distances between H-2K^k antigens and Con A binding sites on murine lymphoma cells. In addition, the spatial relationships of the immunogenic determinants on the antigen itself were studied. The lack of energy transfer between competing anti-H-2K^k antibodies showed that the H-2K^k antigen exists without clustering on the cell surface. Significant energy transfer occurred between Con A molecules, a finding suggesting that the Con A binding sites are clustered on the cell membrane even at 0°C. The Con A binding glycoproteins are in close proximity to the H-2K^k antigens under conditions where the antigens and the binding sites showed dispersed fluorescence pattern. The distances between the binding sites were calculated from the efficiency of energy transfer between non-competing monoclonal antibodies bound to the same H-2K^k antigen energy transfer.

INFLUENCE OF DETERGENTS ON THE SPECTROSCOPIC PROPERTIES OF PURPLE MEMBRANE

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Dye- detergent micellar solutions are closely similar to biological membranes; therefore, they can be used to model processes related to membranes. Light absorption, emission and luminescence polarization of purple membrane isolated from Halobacterium halobium was investigated at 6.3 10^{-6} M concentration as a function of time and detergent concentration, in order to obtain information on the relationship of structure and function of the system. Sodium dodecylsulphate, Triton X-100 and Digitonin were used. The spectroscopic characteristics were checked over a period of 24 hours after starting preparation of the solutions.

New bands appear in the visible region of the absorption spectra after addition of detergents to the solutions. The maxima of the ultraviolet emission spectra originating from the proteins are shifted towards longer waves with increasing detergent concentrations. Simultaneously, photobleaching of the solutions and a decrease in the polarization degree can be observed.

The results can be explained by conformational changes of the protein part of the molecule, an event leading to an increase in the number of unburied tryptophanes fluorescing in the ultraviolet region of spectrum.

ON THE ROLE OF "VICINAL" WATER IN MEMBRANE PERMEABILITY

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The value of transport co-efficients /e.g. L_p, σ, Q^*, etc./ is determined by the microphysical characteristics of interfaces. In the opinion of Ling and Drost-Hansen there is a polarized vicinal water of a thickness of several times 10 nm, showing anomal characteristics, especially at 15, 30, 45°C along the surfaces.

It is supposed that the vicinal water - lining of the pores of cellophane /cellulose/ films with a pore radius of 3 to 8 nm on the average can also show an anomaly. The temperature dependence of the L_p hydraulic conductivity can deviate from the temperature dependence of the fluidity of bulk water. Our measurements were performed on small cellophane bags - fixed to the end of a glass tube of a length of 1 m and strengthened with a tulle net - in distilled water, which was stirred by a magnetic stirrer. Our results are as follows:

1. Taking the average of the data of measurements there is no anomaly in the temperature dependence of L_p; in essential, it agrees with the fluidity of bulk water.

2. But if the L_p values measured from the direction of cold to warm and those measured from the opposite direction are represented apart from each other, a hysteresis is brought about which is particularly expressed at 15°C. In this case the value of L_p is 15 per cent higher in the system formerly warmer than in the system formerly colder.

3. Hysteresis /structure memory?/ can usually be demonstrated also at other temperatures between 10 and 35°C.

This effect - if proved further - can have a far - reaching significance in biology.

INTERACTION OF SOME NON-IONIC SURFACTANTS WITH MEMBRANE LIPIDS

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The homologous series of non-ionic surfactants of practical importance /nonyl-phenyl-, tributyl-phenyl-, oleic-, lauric-, stearic acid-ethylene oxide polymers/ are excellent compounds to study the interaction between membrane lipids and amphiphilic materials.

Summarizing our results obtained by ^{42}K-efflux, DSC and ESR investigations we can establish that surfactants do modify the structure and function of lipid membranes: they decrease the phase transition temperature and enthalpy, alter the correlation time of spin label /12NS/ and increase the permeability of lipid vesicles. These effects depend:

a/ on the chemical structure of the surfactant molecules. Alteration of the number of ethylene oxide groups per molecule /i.e. change of the hydrophilic part/ influences the effects to a greater extent than does the alteration of the hydrophobic part of molecule.

b/ on the polar head group of the lipid. The membrane perturbation is less effective in DPPE /dipalmitoyl-phosphatidyl-ethanolamine/ dispersion where the head group region is packed more closely than in DPPC /dipalmitoyl-phosphatidyl choline/.

c/ on the environment. In the presence of monovalent ions the effects of surfactant will be inhibited, depending on the concentration and type of the ion.

ESR STUDY OF LIPID-WATER SYSTEMS

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In order to study the pretransition in membranes measurements were made using a new spinlabel with nitroxide headgroup and long hydrocarbon chain.

The results show pretransition to appear in the range of 32 ^{o}C - 37 ^{o}C in DPPC vesicles as well as in DPPC dispersion.

This result is also supported by apparent absorption measurements.

The effect of NaCl was also investigated at a concentration of 3 mol/l. The correlation time in the samples containing NaCl is higher than in pure lipid at all temperatures.

The NaCl affects also the transition temperatures.

The temperature range of the pretransition is moved down by about 2 ^{o}C, and the maintransition temperature is increased by about 0.5 ^{o}C.

Results of previous DSC measurements show similar tendency.

DATA ON THE ACTION OF THE CHANNEL FORMING ANTIBIOTICS GRAMICIDIN AND PRIMYCIN ON THE CATION PERMEABILITY OF HUMAN ERYTHROCYTES

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In our earlier work primycin was found to belong to the channel-forming antibiotics. In order to elucidate the exact mode of action we compared the effect of primycin on the erythrocyte membrane's permeability with the effect of the well-known antibiotic gramicidin. For the exchange diffusion of alkali cations the same type of kinetic curves were obtained with both antibiotics. To explain the kinetic results a statistical mechanical model has been developed. According to the model, aggregates of antibiotic molecules are formed in the membrane; and there is a critical concentration of these substances below which unmodified erythrocytes may also exist.

To prove this model the ratio of unmodified erythrocytes was calculated at different gramicidin concentrations. The saturation values of the influx and efflux were used for the calculation.

Comparison of the influx and efflux values revealed that a membrane potential appears in the antibiotic-modified membrane. The values of the membrane potential were also calculated on the basis of radioisotope -kinetic and flame photometric measurements.

EFFECT OF SOME CARDIOACTIVE DRUGS ON THE CATION TRANSPORT OF THE RED BLOOD CELL MEMBRANE OF HEALTHY AND HYPERTHYROID PERSONS

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Metothyrin is widely used in the treatment of hyperthyri hyperthyroidism and Propranolol is applied for prevention of its side-effects, e.g. arrhythymia and tachycardia. To elucidate the origin of the experienced advantageous effect we investigated the effect of these compounds on the active and passive cation transport. The active transport inhibition of the red blood cells due to excess T_3 and T_4 in hyperthyroid patients was decreased by Methothyrin + Propranolol treatment and the passive transport somewhat increased. Without Metothyrin,even high doses of Propranolol failed to change the in vitro or in vivo red blood cell cation transport of euthyroid and hyperthyroid persons.

However, in case of in vitro Propranolol treatment in the presence of Ca, the active transport decreased considerably as a function of Ca concentration, while the passive transport slightly increased. If only Ca was applied, the active transport decreased to the same degree, but the passive transport remained unchanged. It might be assumed that the effect observed is due to the Na-K ATP-ase inhibitory action of Ca. The permeability increase manifested in the enhanced passive transport may be attributed to the simultaneous effect of Propranolol and Ca.

According to clinical experiences, Digoxine has a weaker therapeutic effect in hyperthyroid than in healthy patients.

Seeking an explanation for this we investigated the alkali ion transport of euthyroid and hyperthyroid persons treated with Digoxine.

In case of hyperthyroid patients the Rb^+ (K^+) active transport of red cells decreased, while the rate of passive efflux increased. Digoxine further intensified the altered active and passive transport of Rb^+ (K^+) in the red blood cells of hyperthyroid patients. After in vitro administration of Digoxine both alterations were enhanced as a function of drug concentration. Spin label measurements with normal and modified erythrocytes revealed that thyroid hormones and Digoxine cause a phase separation in the lipid layer of red blood cell membrane. The results indicate that these compounds enter the apolar part of the lipid bilayer, perturbate its structure and increase membrane permeability, i.e. the rate of passive Rb^+ (K^+) transport. As to the active transport an indirect effect of these drugs on the lipid layer should also be taken into consideration, beside a direct inhibitory action on the Na^+ -K^+ dependent ATP-ase activity.

ION-EFFECTS ON THE STRUCTURE OF NUCLEOPROTEINS

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Optical and calorimetrical melting methods have been used to study the structural damages to the genetical substance of a T7 bacteriophage model-system. The structure of the macromolecules is characterized by the melting temperatures /T_m/ which are sensitive to, and measurably changed by, low concentrations ($\sim 10^{-3}$) of modified bases, due to the cooperativity of the phase transition. [1]

The aim of this study was to find the optimal solution parameters /ion conc., pH/ in which the damages to different parts of the nucleoprotein /DNA, proteins/ can be separately detected by the UV absorption melting method.

In TRIS-HCl, NaCl buffer that optimal ionic strength range was determined /0.11-0.15/ where the high-temperature phase transition of the nucleoprotein is separated into three well-distinguishable steps. For good resolution a TRIS-Na^+ ratio of 2 to 1 proved to be optimal; here, the reaction can be shifted to lower temperature. At lower ionic strengths or with other ion components /phosphate buffer/ the steps flow together,wwhile at higher ionic strengths the boiling of the solution will disturb the measurements.

Dependence of the three melting temperatures on the ionic strength and, separately, on the Na^+ concentration has been determined. The second T_m follows the relation found for isolated DNA. [2] Therefore in this transition the protein-free

DNA fragments melt. The structural changes of the proteins and those of the protein-stabilized DNA fragments take place in the first and third steps. At the same ionic strength the changes in NaCl and those in TRIS exert different influence on the melting profile and temperature, i.e. on the conformation of the macromolecule.

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4,5',8-TRIMETHYLPSORALEN-NUCLEOPROTEIN COMPLEXES AND PHOTOCHEMICAL REACTIONS

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The influence of TMP /4,5',8-trimethylpsoralen/, used in the PUVA therapy, has been studied on the DNA structure, of T7 bacteriophage. By comparing the intraphage--, heat-disrupted-- /65°C/ and chemically-isolated DNA--TMP complexes we studied the effect of proteins on the reaction on the photoreaction as a function of 355 nm UVA dose. Binding of TMP was detected by changes of the heat-denaturation parameters of the nucleoprotein, measured by optical density /OD/ and CD melting.
Results: The OD /260 and 320 nm/ and the CD /225 and 280 nm/ melting curves show three distinct high-temperature melting steps of the nucleoprotein /in 0.05 mol/l TRIS; 0.1 mol/l NaCl at pH 7/, the first /85°C/ being due to the protein, the second one /90.5°C/ to the protein-free DNA and the third one /94.5°C/ to common denaturation of the protein-DNA. Dark complexation with TMP decreases each of the three Tm values, i.e. it destabilizes the whole system depending on the concentration ratio of TMP to nucleotide base. The photoreaction has a destabilizing effect at low UV doses /< 0.5 kJ/m^2/ while a stabilizing one at higher doses. The photochemical reactions of the isolated DNA and those parts of the nucleoproteins which melt in the course of the second melting step were identical. The photochemical reaction of that part of the nucleoprotein which melts in the third step is enhanced in previously heat-

disrupted phage. In this case a stabilization of the α-helix regions of the protein could also be observed.

STUDY OF INTACT PHAGES AND THEIR ISOLATED NUCLEIC ACIDS ON THE BASES OF THEIR VUV SPECTRA

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A new method has previously been published for the preparation of native, high- quality DNA thin films for optical measurements /A. Fekete, I. Földvári: studia biophys. 73, 47-73 /1978//. A variant of this method can be used for preparing intact T7 and MS2 phage films as well. In the present work the intact T7 and MS2 phage films were investigated in the VUV region and were compared to isolated T7-DNA and MS2-RNA. The VUV spectra of intact phages and those of phages with denaturated protein coat were found to be distinctly different, especially in the region of the Y-band /near 200 nm/. Therefore, the denaturation of the protein coat can be detected by the VUV spectra.

In previous investigations a difference was found in the UV-damage to intact T7 phages and isolated T7-DNA /A. Fekete, Gy. Rontó: studia Biophys. 80, 165-171 /1980//. In the present work the investigations were extended to MS2 phages and MS2-RNA as well, and the spectral changes due to UV-light / λ =254 nm/ were detected in the whole range from 300 to 160 nm. In the case of isolated T7-DNA and MS2-RNA an increase of the absorption near 190 nm was found which is characteristic of pyrimidine dimers and photohydrates. In the spectra of intact T7 and MS2

phages the spectral changes were different, a finding explained by the production of different types of nucleic acid-protein cross-links.

NEW DATA ON THE MODE OF ACTION OF LOCAL ANESTHETICS IN UNMYELINATED NERVES OF THE CRAYFISH: RESULTS OF ESR MEASUREMENTS

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Procaine derivatives spin-labelled at their hydrophobic ends were used. The experiments were performed on unmyelinated nerve trunks dissected from the claw of the fresh-water crayfish Astacus astacus. The potency of the drugs was determined by comparison of their minimal blocking concentration. ESR spectra of the drugs were taken both in solution and in nerve membrane and the rotational correlation times calculated. No strong immobilization was found, but the correlation time was always significantly longer in the nerve than in the solution.

In other experiments nerve membranes were labelled with 5- or 16-doxyl stearic acids, and the procaine-induced change in the order-parameter was determined from the ESR spectrum. Procaine caused a decrease of the order parameter in the case of the 5-doxyl stearic acid, but no change was found in the case of 16-doxyl stearic acid.

CONTRACTION OF GLYCERINATED INSECT FLIGHT MUSCLE AS REVEALED IN THE ELECTRON MICROSCOPE

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The mechanism of muscle contraction was studied in glycerinated insect flight muscle stretched in rigor. The stretch broke several sets of actin filaments at the Z lines, but the continuity of the sarcomeres was maintained by the C filaments. The broken sarcomeres were considered to be muscle models in which the movement of non.-actin-bound filaments could be analyzed, and thus new information be expected on the extent and conditions of the filament movement.

In assymetrical sarcomeres - sarcomeres in which the filaments only break at one of the two Z lines - the actin filaments were completely moved out from the injured side of the sarcomere to the intact side. In our experiemental system, it is most probable that the actin filaments could be slided by also cross-bridges localized in the border of the M region. This means that the cross-bridges can act as "independent force generators" and the actin filaments could be disconnected from the force-producing cross-bridges not only in the direction of elongation but also in the direction of shortening by active processes. On the other hand viscous resistance to the relative sliding motion of myosin and actin filaments is negligible compared to the forces produced by one or two cross-bridges. In symmetrically broken sarcomeres, zones of overlapped actin filaments developed in the middle of the A bands. Although half

of the cross-bridges was connected to the actin filaments, the further movement of them was inhibited at the overlapping zones. On the basis of these findings, it is supposed that the opposite polarity of the overlapped actin filaments induces repulsive forces which can equilibrate quite strong contractile forces. If this hypothesis is correct then the striated muscles will be actually shortened only as far as a complete overlapping zone of the actin filaments develops and the further shortening will be prevented by the molecular interaction of oppositely polarized and completely overlapped actin filaments. Our recent findings are in good agreement with earlier observation on mechanical work of the muscle.

EFFECT OF ILLUMINATION ON THE ELECTRIC RESISTANCE OF FROG MUSCLE MEMBRANE

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Previous experiments in this laboratory showed photosensitized sciatic nerve of the frog to produce repetitive response when stimulated with a single electric pulse during illumination by visible light. The transmembrane resistance of oxycholesterol BLMs decreases upon the effect of strong illumination; the time constant of the decrease is a few ms.

The present experiments were performed on superficial fibers of frog /Rana esculenta/ m. sartorius without any photosensitiser. A double microelectrode voltage clamp setup was used to polarize the cellular membrane and to measure the change in the membrane conductance in response to illumination by visible light. Illumination of the muscle increased the conductance of the hyperpolarized membrane in a dose-related manner. This "membrane-photoeffect" can be modelled by a photodiode, too. All these give new experimental support to the semiconductor hypothesis of excitatory processes.

ANALYSIS OF COMPOUND UNIT POTENTIALS

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The analysis of the compound surface EMG records in directed to obtain information on the activity of the motor units. The aim of the present work is to contribute to investigation of the mechanism figuring in the summation of different unit potentials.

The experiments were carried out on frog's nerve-muscle preparations representing "arteficial motor units" /Partridge, L.D.: J. Appl. Physiol. 20 150-156, 1965/. The nerve trunks separated from the sciatic plexus were stimulated separately and also simultaneously, and the action potentials of the muscle were analyzed.

The results revealed a linear summation of the electrical activities generated by the "arteficial motor units" of the muscle.

K^+-EFFLUX IN MUSCLE DURING GLYCERINATION

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Frog sartorius muscles were immersed in 50% glycerol solution at -13C^{o} and the time-dependence of K^+-efflux was investigated for five days. One of the sartorius muscles was put in an ion-free 50% glycerol solution. The other sartorius of the same frog was placed in 50% glycerol solution containing 55 mM NaCl. In the first six hours of glycerination the K^+-efflux was significantly higher in the muscle put in Na^+-containing glycerol. After the fast changes of K^+-efflux in the first phase of glycerination the K^+ remained in muscle decreased in equal fashion in both sartorii, i.e. both of them showed similar exponential curves with equal rate constants. The difference in K^+-content found at the beginning of glycerination persisted, not markedly decreasing but after the 4th day. The analysis of exponential curves revealed that about 10-15% of muscle potassium was released fastly in the presence of Na^+. Thus it can be concluded that part of muscle potassium participated in a K^+/Na^+ ion-exchange, an event that could play an important role in the fast excitation processes of the muscle.

CONTRACTION DUE TO DOMAIN ROTATION OF ACTIN-CONTAINING FILAMENTS

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The time-resolved low-angle X-ray diffraction patterns give the time-averaged position of the molecular components of the muscles, whereas the EPR spectroscopy is sensitive to the frequency of the motion too. Labelling the SH_2 groups of myosin in glycerol-extracted muscle fibres we found that, during the contraction, the estimated rotational correlation time of the myosin heads is longer than 1 ms. On the other hand, the estimated correlation time measured in F-actin gel is about 10^{-5}-10^{-4} s. Thus, we propose a molecular picture for the thin--thick filament interaction, which is compatible also with the sliding theory. We suppose that the energy liberated during ATP hydrolisis is, in part, transferred to a domain of the thin filaments and changes the conformation and the rotational mode. In connection with the actin-myosin interaction this would result in the sliding of the filaments past each other, in the elevation of the common energy level of the thin-thick filament system and in the dissipation of the energy with an actual relaxation time also depending on the mechanical work to be done by the fibres. The energy dissipation, thus the apparent rate of ATP hydrolisis, could be regulated by changes: in the extent of the activated thin filament domain; in the amount of energy re-transferred to the myosin filaments; in the conformation of the myosin light chains; in the extent of the overlapping region.

INVESTIGATION OF Ca-LOCALIZATION IN HEART-MUSCLES BY ELECTRON MICROSCOPIC AUTORADIOGRAPHY

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The authors have continued their investigations on the Ca content of frog heart muscles.

Investigated was the distribution of Ca over myofibrils and interfibrillar space in the ventricle, auricle and sinus node of the heart.

Grain densities over the myofibrils and the interfibrillar spaces were identical in the muscles and sinus nodes, but significantly higher over the interfibrillar spaces of auricle muscles than over the myofibriles.

An opposite grain distribution was found in the ventricle, muscles where the grain densities were significantly higher over the myofibrils.

The grains over interfibrillar spaces could be found over every cell component /mitochondrium, nucleus, etc./, but significantly more grains were seen over the tubulus of sarcoplasmic reticulum than in other places.

The results obtained agree with the data in the literature concerning Ca storage.

THERMOLUMINESCENCE OF SYNTHETIC CALCIUM-HYDROXYAPATITE AS A MODEL FOR DATING TOOTH ENAMEL

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The human tooth enamel is an appropriate system for biological application of thermoluminescent dating /TLD/, because of its low organic content and simple structure.

In order to qualify the tooth enamel from the point of view of TLD, I examined the thermoluminescence properties of synthetic calcium-hydroxyapatite /CHAP/, since CHAP is the essential inorganic component of tooth enamel.

The peak distribution of the glow-curve of CHAP /60 ^{o}C, 285 ^{o}C, 440 ^{o}C/ and the great sensitivity of the peak at low temperature show the possibility of a pre-dose principled measuring.

In order to describe the TL process responsible for the peak of 60 degrees I carried out a computer fitting of the different model-functions to the glow-curves obtained from the experiment.

The continuous model, obtained from the examination, describes well the displacement of the peak position experienced at the isothermal decay, and it is in agreement with the non-monoexponential character of the experimental decay curve. The shape of the experimental decay curve is dose-dependent. The results refer to a trap-structure which confirms the applicability of the pre-dose principle.

ULTRASONOGRAPHY AND COMPUTERISED TOMOGRAPHY IN THE PREOPERATIVE EVALUATION OF MEDIASTINAL TUMORS

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Ultrasonography /US/ of mediastinal tumors is difficult, because of the shadowing effect of the lung, the sternum and the ribs. Nevertheless, it is possible to detect pathological changes by ultrasound and to characterise their structure, when they are near to the ventral surface of the body and scans are made through the intercostal space, beside the sternum. Computerised tomography /CT/ is not hindered by air or bone, and so may give complementary data to the ultrasonographic findings.

US was performed by B and K 3401 analog compound gray scale equipment and B and K 3402 real time sector scanner. CT was made by Medicor-Pfizer 0100 CT-scanner.

15 cases of mediastinal tumors previously detected by chest X-ray were investigated by US and CT. All of them underwent surgery thereafter. Exact nature of the tumors was verified by histopathological examination. Ultrasound was informative and was in good correlation with clinical findings in 13 cases, while CT in 12 out of 15.

On the base of the present study US and CT seem to be promising non invasive tools in the preoperative diagnostics of mediastinal tumors.

ECHOGRAPHIC MEASUREMENTS IN OPHTHALMOLOGY

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The following measurements can be performed by echo-ophthalmography:

- sagittal axis of the eye
- thickness of the cornea
- depth of the anterior chamber
- thickness of the lens
- sagittal axis of the vitreous
- thickness of retina, choroid and sclera together
- horizontal, vertical and oblique axes of the globe
- size of intraocular pathological formations and their distance from the sclera and from the lens
- thickness of the optic nerve
- thickness of the extraocular muscles
- site of the globe in the orbit
- size of orbital pathological formations and their distance from the
- globe and from the orbital wall.

Advantages of echographic measurements are:

a/ - in comparison with optical methods:
   - opacity of refractive media does not cause any difficulty
   - in small children most of the measurements can be performed without general anesthesia, through the eyelids

b/ - in comparison with X-ray measurements:

- echography is without any danger; therefore, it is suited for children and pregnant women. The duration in time and the number of examinations are unlimited
- measurement by echography is quicker, more simple and more exact.

THE VALUE OF TRANSURETHRAL AND TRANSRECTAL SCANNING IN THE DIAGNOSTICS OF BLADDER AND PROSTATE DISEASES

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Detection of bladder tumors is based on cystoscopy. However, it is not suitable for stating bladder malformations, since deeper parts of the bladder wall as well as the surrounding tissues cannot be visualised by this method. Intravesical ultrasonic scanning is investigated as a complementary procedure of cystoscopy. Transurethral scanning by an endovesical ultrasonic probe allows investigation of mural infiltration and also of the surrounding structures. According to our experiences it is suitable for staging and postoperative follow-up of bladder tumors.

Transrectal ultrasonic scanning is suitable for estimation of prostate size and structure. On the basis of our investigations it proved to be the best non invasive tool for precise measurement of prostate size and has also some informative value concerning structural changes. However differentiation between benign and malignant lesions must be based on histopathological examinations.

EXPERIENCES WITH PARATHYROID ULTRASONOGRAPHY

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Thirty patients were examined for suspected hyperparathyroidism. Possibilities for localizing the enlarged parathyroid/adenoma or hyperplasia/ are discussed. Ultrasonography showed positive findings in 10 out of the 30 cases. Seven of them were confirmed by surgery, one by autopsy. One case was a secondary hyperparathyroidism. In one case the alteration detected by ultrasound examination proved to be struma nodule surgically, the parathyroids were not removed, and hyperparathyroidism remained. In two "pseudonegative" cases the pseudonegativity was due to the retrosternal location of the adenoma.

GRAY-SCALE ULTRASONOGRAPHIC MONITORING OF CYSTIC DISEASES OF THE PANCREAS

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The introduction of gray-scale ultrasonography offered a new method for examination of the pancreas. However, the expectations attached to the early diagnosis of pancreatic tumors - - wich could be still operated upon - were not fullfilled. On the other hand, in case of cystic diseases it is only this method wich gives direct information on the extension and position of the cyst. Recent findings of the late years proved a big number of pseudocysts to occur after acute pancreatitis which do not show any signs in the course of phisical and X-ray examination. With the aid of this non-invasive examination one can control all the patients after acute pancreatitis. Our experiences are in accordance with those data in the literature, according to which 20 percent of pancreatic pseudocysts after acute pancreatitis disappear without treatment, thus no surgery is needed. A further importance of sonography is that the presence of a pseudocyst can be demonstrated with certainty before retrograde pancreatography. The injection of a contrast agent in a cyst is dangerous and can cause an abscess. According to our results the regular ultrasound control of patients with acute exacerbations of chronic pancreatitis is absolutely necessary.

ECHOTOMOGRAPHY IN DIAGNOSING NEONATE AND INFANT INTRACRANIAL PATHALOGIES

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When designing their standard examination technique which they controlled through conventional radiology and CT the authors developed a reliable ultrasonic analytic method which can be used routinely for neonates and older infants, for both screening and detailed objective analysis of flows.

Almost 500 examinations have proved that for this age-group neurosonology is a substitute of full value for CT, which is both more expensive and more complicate to use.

Examination results are objective, can be analyzed well and can be repeated at will as often as required. It has proved quite successful for early diagnosis of intracranial development disorders, intracranial hemorrhage, for longitudinal analysis of primary and secondary hydrocephalus and for determining progression. The examinations can be done in an outpatient clinic, and they require no preparation. They can also be used to monitor the results of shunt implantation required for ventricular dilatation as well as shunt operation itself.

ABDOMINAL ULTRASONOGRAPHY OF PEDIATRIC SURGICAL DISORDERS

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Ultrasonography is playing an increasingly important role in the examination of pediatric surgery pathologies.

It owes its advance in diagnostics not only to the facts that it is painless and has no contraindication but also to the circumstance that much otherwise unavailable information can be obtained with it, which can be of great service to the pediatrician and pediatric surgeon. Its main field of application comprises the traumatic pathologies of the liver, biliary tract, spleen and pancreas, all being very significant examination areas. The method is growing in importance also in kidney and urinary tract diagnostics.

In diagnosing acute abdominal cases it is particularly important for examining non-functioning organs /blocked gall bladder, acute cholecystitis, development disorders among neonates, differential diagnosis of jaundice/.

It is particularly suitable for examining abdominal and pelvic masses. /Abdominal and pelvic tumours, cystic and torquated ovaries, exact analysis of the size and location of renal and pararenal tumours, including location in relation to other organs, as well as follow-ups of the above parameters during therapy. Abdominal abscess can be located, and precise size can be determined./

The paper presents examples of the use of ultrasound for diagnostics covering a period of two years of pediatrix examinatio

PRENATEL ULTRASOUND DIAGNOSIS OF CONGENITAL MALFORMATIONS

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Prenatal diagnosis as a new trend developed from clinical genetics in the past few years. Making use of the possibilities in prenatal diagnosis it ensures not only the evaluation of recurrence risk but also that of fetal health condition in the case of high-risk families.

Ultrasound is a method indispensable in prenatal genetic diagnosis with the help of which the intrauterine developing fetus can be perceived. On the basis of pathomophologic picture direct detection of several malformations is rended possible as well as indirect detection of other changes by the follow-up of the changes of amniotic fluid, placenta, umbilical cord and fetal movements.

In the present paper the authors give an account of the different types of congenital malformations diagnosed by ultrasound at the Genetic Counselling Unit. Not only the process of the examination, but also other forms of prenatal methods are presented. Depending on the severity of the changes revealed, further course of pregnancy is decided on with the agreement of the couple. By induction of abortion of premature labour tragedies in the life of the individual or the family can be prevented. The social benefit of prenatal genetic care should also be taken into consideration.

IMPORTANCE OF ULTRASOUND METHODS IN THE DIFFERENTIAL DIAGNOSIS OF OVARIAN TUMOURS AND THEIR FOLLOW-UP DURING TREATMENT

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The authors studied the value of ultrasonic examination in differential diagnosis of ovarian tumours, prediction of malignancy as well as in the follow-up examinations of patients with ovarian tumours.

The findings of bimanual examination and those of ultrasonic examination were compared with the actual findings during laparotomy.

It was established that preoperative ultrasonic examinations call attention also to such details which cannot be detected by vaginal examination /internal structure of tumours, their consistency/.

Ultrasonic examination is particularly suitable for the follow-up examination of patients with ovarian tumour. The results of accurate and repeated measurements of diameters of ovarian tumours give information on tumour kinetics which information can then be used in the oncotherapy.

ULTRASOUND DIAGNOSIS OF ABDOMINAL TUMORS IN CHILDREN

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More than 800 ultrasound examinations were performed in the past 4 years for diagnosis of different abdominal diseases in infants and children. Benign or malignant tumours were detected in 120 cases.

Echography proved to be useful in the objective determination of tumor size and localization and also in the description of the tumor mass invasion of the surrounding organs and large blood vessels. The analysis of the internal echo-structure of the mass allowed not only solid/cystic distinction, but very often also necrotic changes and calcification to be observed. These data were helpful during radio- and chemotherapeutical treatment of the neoplasm.

Regular echographic follow-up of tumour patients /especially with malignant tumours/ is warmly suggested. Early detection of recurrence or metastases can affect prognosis as demonstrated in some cases.

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ECHOGRAPHY OF ABDOMINAL DISORDERS DURING PREGNANCY

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The widespread use of echography is of great importance in the diagnosis of abdominal disorders coexisting with pregnancy. The maternal disease and the condition of the pregnancy can be examined in the same time without the risk of fetal exposure to radiation. Numerous examinations can be carried out within a short time whenever a compound and a real-time equipment are simultaneously at disposal.

Analysing the 794 examinations of 600 pregnant women, abdominal disorders

were anticipated in 60 /10%/ and

could be revealed in 82 /13.5%/.

Most common findigs were the pathologic alterations of the uterus and ovaria /myoma, simple cyst, cyst-adenocarcinoma, etc./ and - in other abdominal organs - renal, hepatic and bile duct disorders /hydronephrosis, cholelithiasis, choledochal cyst, etc./.

Echography has a particular importance in following up the illnesses known before the pregnancy /dystopic kidney, transplanted kidney, cirrhosis hepatis, etc./.

Echography of abdominal viscera is indicated in pregnancies, when

- abdominal pathology is known before the pregnancy
- a subjective complaint of the woman begins during the pregnancy

- a palpable alteration of the uterus and/or ovaria can be revealed
- a mass is palpable, independent of the pregnant uterus.

BIOPHYSICAL PARAMETERS OF THE HUMAN UPPERARM MUSCLES AND THE HILL'S EQUATION

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We investigated the sequence of events during a single extension and flexion of the forearm. Positive work is done during a concentric contraction whenever the muscle's moment is actiing in the same direction as does the velocity of the movement.

Even nowdays, the majority of research works is made, "in vivo" on isolated muscles in concentric contraction. A.V. Hill showed that in the isotonic case the tension / F / exerted by the muscle depends on the velocity of muscle shortening / v / in response to the electrical stimulus. He found that

$$/ F + a / / v + b / = / F_o + a / b$$

where F_o is the maximum tension, if v=0 /isometric contraction/, and a,b are constant values. Another form of Hill's equation:

$$/ F + a / / v + b / = / v_o + b / a$$

where v_o is the maximum shortening velocity, if F=0. In the above equations the shortening velocity is practically inversely proportional to the force / F /. This can be best seen in the following form:

$$v = b \frac{F_o - F}{a + F}$$

The above parameters of the Hill's characteristic equation were obtained on students and sportsmen. Our

measurements were done on intact human muscles /M.biceps and triceps brachii/. With the help of two transducers the mechanical parameters / F, F_o, v, v_o / were converted into electric signals. The work / W / and power / P / of muscles were calculated by computer.

DETERMINATION OF CYCLODEXTRIN INCLUSION COMPLEX STABILITY BY REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY

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Due to the favourable physico-chemical characteristics of cyclodextrin inclusion complexes of bioactive compounds their rapidly broadening application is expected in the future not only in the human therapy but also in chemical pest control in agriculture. The beneficial effect of complex formation strongly depends on the complex stability. However, the traditional methods to determine interaction /or inclusion complex formation/ energy of cyclodextrins and bioactive compounds are very time-consuming and of poor reproducibility.

Since cyclodextrins are very hydrophilic and the bioactive molecules to be complexed with are lipophilic, complex formation will decrease the lipophilicity of the molecules included in the cavity of cyclodextrins.

This phenomenon was exploited in our method: we determined the lipophilicity of the bioactive compound first in an appropriate reversed-phase thin-layer chromatographic /RPTLC/ system free of cyclodextrin and then in the same RPTLC system with added cyclodextrin. The differences in lipophilicity were related to the complex stability. The results of our method are in good agreement with the results of other generally accepted methods.

STUDIES OF THE MUTAGEN ACTIVITY OF CHEMICALS IN BACTERIOPHAGE T7

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Various tests for mutagenicity and carcinogenicity can be found in the literature. There are only few examples where the test applied gives quantitative characterization of the mutagen activity of the substances. In our test the DED-value /dimer equivalent dose/ defined by us earlier is used to specify the probability of mutation in unit concentration of the solution tested. Quantitative results of other tests described in the literature were summarized by Parodi et al. /1982/.* A series of substances was studied by our method and compared with the indices of other mutagen tests published by Parodi. The general agreement demonstrated by the experimental data has outlined the applicability of the quantitative method developed by us. The cause of deviations found in some cases are analyzed.

*Quantitative correlations amongst alkaline DNA fragmentation, DNA covalent binding, mutagenicity in the Ames test a carcinogenicity, for 21 compounds.
Parodi et al. Mut. Res., 93 /1982/ 1-24.

THE DARK-BINDING OF 4'-AMINOMETHYL-4,5',8-TRIMETHYLPSORALEN TO DNA AND NUCLEOPROTEINS

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Psoralen-derivatives have been used in psoriasis-therapy as UV photochemotherapeutic agents. Recently, new derivatives were synthesized by Isaacs et al. /1/, among which 4'-aminomethyl-4,5',8-trimethylpsoralen /AMT/ has the special advantages of having a much higher association with DNA and a high solubility in water. In our work we studied how the dark binding /association/ of this molecules to DNA is affected by the presence of proteins. To study dark binding we assumed a two-step reaction with the substrate /DNA/ according to the following scheme:

$$\text{AMT} + \text{DNA} \overset{K_1}{\rightleftharpoons} \text{complex I} \overset{K_2}{\rightleftharpoons} \text{complex II}$$

where complex I is stabilized by Coulombic forces, complex II is a stacked complex, K_1 and K_2 are the binding constants for the two reactions. It can also be assumed that the quantum yield of fluorescence of AMT is not affected within complex I, but it is quenched within complex II /2/. This model leads to the following relation:

$$\frac{1}{1-R_H} = 1 + \frac{1}{K_2} + \frac{1}{K_1}\frac{1}{K_2}\left[\frac{1}{P}\right]$$

where R_H is the ratio of the mean quantum yield of completely isolated bound AMT and the quantum yield of free ATM; [P] is the molar phosphate concentration for DNA. In our experiments R_H was directly measured, and $\frac{1}{1-R_H}$ plotted against $\left[\frac{1}{P}\right]$ gave the

constants K_1 and K_2. The buffer used was: 0.01 mol/l tris, 0.01 mol/l NaCl, 0.001 mol/m EDTA, pH 8.5 to assure the electrostatic binding. The case of the nucleoprotein; the bacteriophage T7 was compared with the case of DNA calf thymus. The results showed that the binding constants are not strongly affected by the presence of coat proteins. This conclusion is in agreement with our optical melting results demonstrating that in the buffer used the structure of intraphage DNA is not strongly affected by the presence of coat proteins.

1./ S.T. Isaacs, Ch.J. Shen, J.E. Hearst, H. Rapoport: Biochemistry /1977/ 16, 1058-1064.

2./ J.-J. Toulme, C. Heléne: J. Biophys. Chem. /1977/ 252, 244-249.

INTERACTIONS OF MONO- AND DIVALENT CATIONS IN PLANTS

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Winter wheat seddlings /Triticum aestivum L. cv Martonvásári 8/ were grown in tridistilled water, 0.5 mmol dm^{-3} $CaSO_4$ solution and complete nutrient solution with different K^+ supplies. The K, Na, Mg and Ca contents of roots and shoots were determined daily between the 7th and 11th days /from one-leaf stage to two-leaf stage/. The analysis of the Na=f/Ca/ and D=f/M/ state-sets /where M=K+Na and D=Mg+Ca/ led to the following conclusions:

1. By the 7th day shoots and roots reached one of the ionic states as follows; deficient ionic state /M and D are low/, low ionic state /M is low but D is high/, high ionic state /M is high but D is low/.
2. The mechanisms of the uptake and regulation of K, Na /M/ and Mg, Ca /D/ pairs seem to be different, allowing an interaction /competition/ between the ions in each pair. The monovalent and divalent cations /M and D/, however, are also mutually affected, which can be described by the empirical relationship:

$$(D - D_{opt.})^2 = C_1(M - M_{opt.})^2 + C_2,$$

where C_1, C_2, $M_{opt.}$ and $D_{opt.}$ are constants.

3. On the basis of the Gouy-Chapman theory of electric double layers, an average surface charge density (σ) and surface

potential (ψ_o) can be attributed to the cell constituents of shoots and roots in every ionic states. Changes in σ and (ψ_o) enable the seedlings to move, under appropriate outer conditions, their ionic state near to $M_{opt.}$; $D_{opt.}$. This can be a fabourable ionic state for enzyme reactions and relative growth rate.

4. Due to water uptake during the germination M and D contents of seeds /in μmol per g fresh weight/ decrease and $M-M_{opt.}$ and $D-D_{opt.}$ signals can appear providing an appropriate stimulus for active mechanisms of ion uptake. Thus, /$M_{opt.}$; $D_{opt.}$/ state seems to be coded by the structure and ion contents of seeds.

HORMONAL REGULATION CF Ca TRANSPORT

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Active Ca transport of cells /uptake, storage, release/ is carried out by membrane-bound Ca-ATPase. Activity of ion-dependent transport ATPases can be modified by hormones /Erdei, L., Tóth, I. and Zsoldos, F. 1979. Physiol. Plant. 45:448-452./. The activation of the microsomal Ca-ATPase from roots of winter wheat seedlings, grown in complete nutrient solution and pretreated with benzyladenine for 7 days, was studied. As a response to the hormonal treatment /1/ the ion-independent basic ATPase activity increased, /2/ the affinity of ATPase towards Ca^{2+} increased, /3/ calmodulin prepared from either roots of winter wheat or from erythrocytes stimulated Ca-ATPase activity, /4/ calmodulin from both the hormone-pretreated and untreated seedling's roots were effective, while only membranes from hormone-pretreated plants were responsive, and /5/ the hormone-pretreated seedlings contained less Ca than the untreated plants.

In conclusion, benzyladenine can induce de novo synthesis of high affinity binding sites for Ca: calmodulin complexes which can interact with the Ca-ATPase in the plasmalemma. This can lead to a more efficient Ca-extrusion system, maintaining the cells in juvenile form.

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FACILITATION OF ^{45}Ca TRANSPORT ACROSS PLANT MEMBRANES BY COMPLEX-FORMING AGENTS

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The process of calcium transport across plant epidermis was examined by using ^{45}Ca and liquid scintillation counting. The transport could be facilitated by compounds AB18 and AB20. It was verified that these carrier molecules formed complexes with calcium and carried it acros the membrane in this way.

The mechanisms examined may serve as a good model for solving some practical problems such as research of new types of carrier molecules to be used in plant protection.

CHARACTERIZATION OF THE MACROMOLECULAR TRANSPORT IN CEREBRAL MICROVESSELS

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Cerebral microvessels have peculiar permeability properties: they may represent a barrier to the penetration of certain substances, whereas they could also facilitate the transport of some other materials. Cerebral microvessels are impermeable to macromolecules, thus the circulating serum albumin could not enter the central nervous system under normal conditions. In pathological circumstances, however, serum proteins can reach the brain tissue accross the microvessels of the brain and contribute thus to the pathogenesis of brain oedemas of vasogen type.

The authors studied with immunohistochemical and spectrophotometric techniques the characteristics of albumin transport after intracarotid infusion of histamine and after dimethylsulphoxide treatment /BROADWELL et al. Science, 217:164-166, 1982/. The results suggest that, in these circumstances, the macromolecular transport is realized by transendothelial pinocytosis, an active process, in which the metabolism in the capillary wall of cyclic nucleotides plays an important part.

ENERGY-VALVE FUNCTION OF CAROTENOIDS IN PHOTOSYNTHESIS

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Carotenoids have four functions in the photosynthetic apparatus of green plants. They are:

- antenna pigments absorbing in the spectrum region where chlorophylls are inefficient and transferring their excitation energy to chlorophylls,
- photoprotective agents preventing chlorophylls from the harmful effects of singlet oxygen and removing the excitation energy from the chlorophylls by triplet-triplet energy transfer,
- structural elements in the architecture of the thylakoid membrane,
- constituents of the xantophyll-cycle.

From measurements of chlorophyll fluorescence induction in wide time-range / $\mu s \longrightarrow s$/ we attampted to decide which of the first two functions is predominant considering physiological and evolutionary points of view. Therefore, our measurements were carried out both on wild type and on carotenoid-deficient mutants of a higher plant /maize/. Analysis of the kinetics of the fluorescence induction revealed a surprisingly high rate of excitation energy transfer from the antenna to the reaction center even in the mutants which are immediately photokilled at higher /about 10 Wcm^{-2}/ light intensities.

The results suggest that the photoprotective role of carotenoids was of utmost importance in the ancient time /under reducing atmosphere/ and is persisting even now /under oxidizing atmosphere/.

UNUSUAL CHARACTER OF PHOTOSYNTHETIC ELECTRON-TRANSPORT CHAIN IN GREENING BARLEY LEAVES TREATED WITH PYRIDAZINONE HERBICIDES

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Pyridazinone herbicides are known to inhibit the photosynthetic electron-transport in isolated chloroplasts as well as in algae. In our experiments etiolated barley leaves were allowed to green in the presence of pyridazinone herbicides /SANDOZ 6706, 9785, 9789/. The photosynthetic activity of the leaves was studied by recording the fluorescence induction curves of the intact leaves and by measuring the photochemical activities of the chloroplasts isolated from the leaves. In the latter case the activities of electron-transport chain from water to ferricyanide, from water to methylviologene and from reduced dichlorophenol-indophenol to methylviologene were determined with a platinum electrode sensitive to oxygen in the presence and absence of an uncoupler. Our results show that the photosystem 2 of the treated leaves is inactive in function, while the photosystem 1 has an activity that nearly equals the control. The treated leaf chloroplasts, however, exhibited a surprisingly high activity from water to methylviologene, and this activity was found to be insensitive to dichlorophenyl--dimethylurea. It is suggested that the coupling between the electron transport and photophosphorilation can be considered as an essential regulatory factor in the function of the photosystems.

UNUSUAL PHOTOSYNTHETIC OXYGEN EVOLUTION

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Though Chlorella membranes show profound changes after cerulenin[*] treatment, the O_2-evolving system remains intact. Even more surprisingly, dichlorophenylurea /DCMU/-insensitive O_2 evolution has been found, an event suggesting an unusual mode of O_2 evolution. In order to clarifiy this mode of evolution polarographic studies were performed under continuous and flashing light. For the usual way under flashing light, a sudden burst and an oscillation of O_2-emission with a period of four flashes appear and are explained in terms of the Kok model, in which the O_2-evolving systems do not interact. In the unusual mode under flashing light no burst or oscillation were observed, while under continuous light the evolution rate remained practically unaltered. This suggests that the new mode of evolution is a result of cooperation between the O_2-evolving systems. This mechanism may play a role under normal photosynthesizing conditions and should be taken into account in explaining anomalies of O_2-evolution, especially in cases of herbicide resistance.

[*]An inhibitor of the de novo biosynthesis of fatty acids

SPECTROSCOPY OF LONG-WAVELENGTH FORM OF PROTOCHLOROPHYLL IN SOLID FILMS

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Long-wavelength forms of protochlorophyll /PChl/ were prepared in solid films on glass surface by water vapour treatment of the films. A 48-hour treatment at room temperature resulted in the appearance of the main absorption maximum of the films at 677 nm and there appeared a shoulder at about 650 nm. The fluorescence emission spectra showed maxima at 655, 693 and 707 nm when the excitation was carried out at 470 nm. Absorption and fluorescence bands in similar positions could be observed in the spectra of etiolated leaves and of pumpkin and Cyclanthera seed coats. The circular dichroism /CD/ spectra of the water-treated films exhibited very intense CD signals: strongly asymmetrical positive Cotton effect appeared around 650 nm /a small negative signal at 642 nm, zero crossing at 650 nm and v very intense positive signal at 667 nm/. The shape and the high intensity of the CD signals are indicative of the presence of a PChl form with definite "crystallic" geometry. In addition to this form there are PChl forms in the films which have fluores fluorescence maxima at 693 and 707 nm and have no CD signals. These forms probably have a non-definite or "random" geometry.

It is not excluded that similar, long-wavelength forms exist also in vivo, the molecular arrangement of these forms can probably be similar to that of forms observed in solid films.

FLASH-INDUCED OSCILLATION OF THERMOLUMINESCENCE IN ISOLATED CHLOROPLASTS

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It is known that thermoluminescence /TL/ of chloroplasts is connected with the reversal of certain steps of the charge separation process in PSII. The correspondence of TL components to known donors and acceptors of the electron transport chain is however contradictory in the literature.

To investigate this question thermoluminescence of isolated maize chloroplasts was measured using flash excitation. It was found that, in contrast to the literature, the amplitude of Z_v band - which can be measured between $-80^{o}C$ and $-30^{o}C$ - shows a four-period oscillation with maxima at the 0th, 4th and 8th flashes. The B band at $+30^{o}C$ also oscillates with a period of four showing maxima at the 2nd, 6th and 10th flashes. With increasing dark adaption time the oscillatory pattern will be changed. After 2-hour dark adaptation the maxima are shifted to the first, 5th and 9th flashes. Further extension of the dark period results in the appearance of a two-period contribution in the oscillatory pattern with maxima at uneven number of flashes. After DCMU addition the B band is replaced by a band at $+10^{o}C$ with oscillatory behaviour similar to that of the B band. In the presence of DCMU a band can also be measured at $+45^{o}C$ showing a two-period oscillation with maxima at the first,

3th, 5th and 7th flashes. The experimental results were compared with the results of a computer assisted model calculation in which positive charges were supplied by the S states of the water splitting enzyme and electrons by the primary /$\underline{O}$/ and secondary /B/ acceptors. It was concluded that the Z_v band is related to a donor which is in equilibrium with the S_1 state and to an acceptor in front of the DCMU inhibitory site. The B band oridinates in the recombination of S_2B^- and S_3B^- and the band at $+10^oC$ can be attributed to the recombination of $S_2\underline{O}^-$ and $S_3\underline{O}^-$. As to the band at $+45^oC$ it can probably be related to the primary acceptor $\underline{O}^-$ and a donor which is in charge exchange with the S_o and S_1 states.

FLOW CYTOMETRIC ANALYSIS OF VIABILITY OF MAMMALIAN SPERM CELLS

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Artificial insemination is a widely applied and in some cases almost exclusive technique in animal husbandry. Among the various factors influencing the result of the insemination the quality of the sperm plays a vital role. The classic way of qualifying a sperm sample in the veterinary practice has been the microscopic investigation.

Fluorescein diacetate and propidium iodide double fluorescence labeling of animal seminal fluids in combination with 55°C treatment of various times /5-60 min/ was used to monitor the viability and heat tolerance of sperm cells by flow cytometry. The great advantage of flow cytometry is the accuracy of the evaluation of the test since a large cell population can be investigated in a resonable short time.

Good correlation has been found between the results of the above mentioned test and the known fertility values of bulls. The method seems to be suitable to test the fertility of other male animals as well.

The simplicity of the relatively cheap procedure may provide a new screening method to qualify male animals before using their seminal fluid for large scale artificial insemination.

REGULATION OF THE BACTERIORHODOPSIN PROTON PUMP, PHOTOELECTRIC GENERATOR "IN VIVO"

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Time-resolved absorption spectroscopy has been used to measure the halftime decay of the bacterirohodopsin M_{412} photocycle intermediate $/M_{\tau 1/2}/$ in anaerobic H. halobium cell suspensions. Under conditions routinely used for such measurements in purple membrane suspensions we found that in cells the decay time changes with the intensity of the actinic flash and, if multiple flashes are used, with the flash frequency. Sensitivity to the uncoupler CCCP indicates that this is due to an increase in the transmembrane potential caused by the actinic flashes. Extensive modification of the flash spectrometer allowed $M_{\tau 1/2}$ measurements to be made with single flashes of low intensity and/or at very low repetition rates. The dependence of $M_{\tau 1/2}$ on $\Delta\psi$ has been examined under conditions where Δ pH = 0. $\Delta\psi$ was monitored by ^{3}H-TPMP^{+} accumulation measured using flow dialysis. CCCP titration experiments show that $M_{\tau 1/2}$ and $\Delta\psi$ are coupled in the cell. Since the decay kinetics are multiexponential and the ratio of the components varies with $\Delta\psi$, a more detailed study will be necessary before the physical mechanism of the coupling between $M_{\tau 1/2}$ and $\Delta\psi$ can be elucidated.

THE KINETICS AND MECHANISM OF BACTERIORHODOPSIN PHOTOCYCLE ARE CONTROLLED BY MEMBRANE POTENTIAL

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Under illumination bacteriorhodopsin undergoes a photocycle and pumps protons across the cell membrane. The membrane potential created by the proton pump has a feedback toward the photocycle /see abstract of Dancsházy, Helgerson and Stoeckenius/. The aim of the present work was to study this feedback effect quantitatively.

The experiments were carried out on cell envelope vesicles, the potential of which was controlled by the intensity of a background illumination. The membrane potential was measured by ^{3}H-TPMP^{+} flow dialysis. Photocycles were synchronously triggered by low intensity laser flashes and the kinetics of the M form of photocycle was followed by measuring absorbance changes. Exponential fitting was used to analyse the kinetical curves. Good fitting was achieved using two exponentials. Their time constants and the ratio of their amplitudes strongly depended on the membrane potential. Two M-like forms were supposed to explain the existence of the two exponentials. The fitting parameters were quantitatively analyzed in the framework of two models, assuming sequentially

and paralelly decaying M forms. The experimental data satisfy only the parallel model. The slowing-down of the decays in the presence of membrane potential was explained by an increase of activation enthalpy whith an additive electrostatic force. This model described correctly the membrane potential dependence of the time constant of one form but that of the other one as well as the ratio of the amplitudes showed some anomalies.

THE PHOTOCHEMICAL CYCLE OF BACTERIORHODOPSIN AS AN INDICATOR OF MEMBRANE POTENCIAL "IN VIVO"

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In the bacteriophodopsin /BR/ photochemical cycle the M_{412} intermediate half decay time [$M_{\tau 1/2}$] is governed by the membrane potential ($\Delta\psi$). The $M_{\tau 1/2}$ undergoes a 8 to 10 - fold increase with increasing $\Delta\psi$. On the basis of this correlation the rise and decay time of $\Delta\psi$ can be determined "in vivo". The $M_{\tau 1/2}$ measured after switching off illumination of different durations /5 - 10 ms/ reflects the relative value of $\Delta\psi$ generated by BR during the illumination.

In this way the rise time of $\Delta\psi$ can be determined. The decay of $\Delta\psi$ can be measured by applying test flashes with different delay times after switching off a continuous light pulse long enough to produce a constant $\Delta\psi$.

EFFECT OF pH ON THE FUNCTION OF THE BACTERIORHODOPSIN PROTON PUMP

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The functioning of bacteriorhodopsin isolated from Halobacterium halobium strongly depends on the proton concentration of the medium. We investigated, how the kinetics of the photocycle, the kinetics and distance of the proton transport depended on pH. Our measuring object was a water suspension of purple membrane fragments containing the bacteriorhodopsin. The membrane fragments were oriented in electric field and the orientation was preserved in gel. Following an exciting laser flash, the photocycle was followed by absorption kinetic measurements and the charge movement measured in the form of displacement current. Since in the whole pH range the measurements were made on the same sample the changes in the distances of charge movements could be followed, in addition to the kinetic analysis. It was found that the details of pumping change strongly differ above and below pH = 8.

STUDY OF THE ELECTRIC SIGNS FROM DRIED ORIENTED PURPLE MEMBRANE SAMPLES

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The author measured the electric and optical responses to a laser flash of dried oriented purple membrane samples prepared from Halobacterium halobium. Studied were the dependence of the signals on the humidity and temperature of the sample and on the external electric field. The activation enthalpies of the proton motion over the energy barriers in the protein during the photocycle of the bacteriorhodopsin was calculated from the temperature dependence of the life times of the signals. The width of the barrier was calculated from the changes of the signals caused by the external electric field. Measurements at different relative humidities allowed assumptions to be made on the role of water in the functioning of bacteriorhodopsin.

POTENTIAL OF CHARGES EMBEDDED IN BIOLOGICAL MEMBRANE VESICLES

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Considering their electrical properties, the closed biological membranes and the artificial lipid vesicles can be modelled by a spherical dielectric layer surrounded by electrolyte.

Electrical potential profiles of the charges originating from a charge separation in the membrane were computed at different ionic strengths of the electrolyte.

We investigated /i/ the configuration of the electric field in the membrane and /ii/ the variations of the potential and ion concentration in the electrolyte along the membrane surface.

Our calculation can be applied to the /i/ interpretation of the kinetics of the electrochromic absorbance change of the pigments embedded in the photosynthetic membrane and /ii/ investigation of the role of local proton- and other ion-concentration differences in bioenergetical processes.

REGULATION OF THYLAKOIDS ENERGIZATION. I. THE PHOTOSYSTEMS

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Participation of the photosystems in the energization of thylakoids was studied by measuring the electrochromic absorbance change of isolated chloroplasts and leaves of higher plants.

The turnover rate of the photosystems was calculated from the amplitude change of the electrochromic absorbance which depends on the intensity of the monochromatic background illumination. It was shown that the turnover rate of photosystem I is much lower than could be expected from the rate of the linear electron transport or, of the slow rise in the electrochromic absorbance change. It is suggested that the slow turnover rate originates from the cyclic electron transport around photosystem I, while the slow rise of the absorbance change originates from the linear electron transport near photosystem I.

REGULATION OF THYLAKOID ENERGIZATION II. EFFECT OF CO_2 ON LEAVES

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Energization of the thylakoids in situ was studied by means of flash-induced electrochromic absorbance change in leaves of higher plants.

It has been shown that CO_2 regulates both the building-up of the energized state of thylakoids and the ATP synthesis. These effects can be accounted for by CO_2-regulated changes in the photosynthetic electron transport and in the membrane permeability.

STUDY OF POSSIBLE CONNECTION OF HYDROGENASE TO THE PHOTOSYNTHETIC ELECTRON TRANSPORT CHAIN IN PHOTOBLEACHED ANABAENA CYLINDRICA

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Photobleached Anabaena cylindrica shows an increased O_2 and H_2 production rate when compared to untreated filaments. Previous experiments demonstrated that a reversible hydrogenase induced during photobleaching was responsible for the enhanced H_2 production. Considering the possible connection of the hydrogenase to photosynthetic electron transport chain, we made the following experiments:

- study of Photosystem I and Photosystem II activities in vivo with the aid of O_2 uptake and production measurements, respectively,
- measurements of fluorescence emission spectra and polarization to check intactness of phycobilisomes,
- measurements relating to the site where hydrogenase connects to one of the components of photosynthetic electron transport chain /electrochrom-shift, electron flow upon addition of artificial electron donor and acceptor/.

ORIENTATION OF HYDROGENASE ENZYME IN VARIOUS PHOTOSYNTHETIC BACTERIA

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The localization and orientation of hydrogenase /E C class 1.12/ in seven strains of photosynthetic bacteria has been determined. All of the tested bacteria possessed hydrogenase activity. Cell membranes of representatives of the Chromatiaceae, Rhodospirillaceae and Chlorobiaceae families were found to be impermeable to the oxidized redox dyes Methyl Viologen and Benzyl Viologen while the reduced forms could easily penetrate the membranes. This permeability difference rendered possible the localization of hydrogenase enzyme since viologen dyes are electron donors and acceptors for the enzyme. Members of the Chromatiaceae and the Rhodospirillaceae contained a predominantly or exclusively membrane-bound enzyme. In contrast, the majority of hydrogenase activity of Chlorobium limicola f. thiosulfatophilum was in the cytoplasm. Hydrogenase or at least its active center was oriented toward the outer surface of the cell membrane in purple bacteria.

Formate - hydrogen - lyase was detected in Rhodospirillaceae, whereas C. limicola showed both formate hydrogen lyase and pyruvate hydrogen lyase enzyme activity. These enzymes interfere with the reaction of hydrogenase and viologen redox dyes; therefore, the exact orientation of hydrogenase in these bacteria needs confirmation by further methods independent of any such interference.

BOUNDARY LIPIDS IN LIPID/PROTEIN RECOMBINANTS

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The ESR spectra of freely diffusible spin labelled lipids in lipid/protein recombinants consist of two components: a motionally restricted one and a rapidly diffusing one. From the quantitative evaluation of these two-component spectra the binding stoichiometry can be calculated, and further information can be obtained concerning the lipid head-group selectivity and the exchange rate between boundary and bulk lipid sites. Saturation transfer ESR provides additional data as to the rotational diffusion of membrane proteins. This novel spin labelling method will be illustrated with our data on the myelin proteolipid protein/dimiristoyl phosphatidylcholine interaction.

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PROTON CONDUCTION IN LIPIDS

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The semiconducting properties of lipids in different hydration states were investigated. Electrode effects which are indicative of ionic processes were established irrespective of the nature of lipids and the level of hydration. Thermoelectric measurements indicated positive charge carriers to be involved in charge propagation process. Electrolysis experiments provided direct evidence for proton conduction.

Activation energies of conduction were determined under various conditions. The activation energy in fully hydrated state was found to approximate to that of water or ice. A conduction mechanism based on lipid head group motion and water cluster formation is proposed.

INSTRUMENT DEVELOPMENT IN THE INSTITUTE OF BIOPHYSICS OF THE BIOLOGICAL RESEARCH CENTER IN SZEGED

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We try to demonstrate the activity of the Instrument Development Group. With help of photos and short specifications we give examples of the last years' well-proved products.

INTELLIGENT FLASH PHOTOLYSIS MEASURING SYSTEM

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The intelligent flash photolysis measuring system, developed in the Institute of Biophysics of the Biological Research Center, serves to follow chemical reactions excited by light flash.

Main components:

i. Exciting system /N_2 laser or dye-lasers/

ii. Optical measuring system /Xe arc lamp stabilized to 0.1%, monochromators, photomultiplier detection/

iii. Signal measuring and processing system /microprocessor - controlled CAMAC system/

Main characteristics:

- automatic
- programmable
- on-line data processing
- 100 ns minimal channel time
- 10^{-5} error
- 220-700 nm optical measuring range.

X-RAY MICROANALYSER SYSTEM WITH A POSSIBILITY OF INDEPENDENT MASS MEASUREMENT AND ITS APPLICATION FOR EXAMINATION OF BIOLOGICAL SAMPLES

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An X-ray microanalyser assembly is demonstrated, the main parts of wich are an EDAX 183B semiconductor detector and a KFKI ICA 70 4k multichannel analyser. The microanalyser system is attached to a JEOL 100 B transmission electron microscope.

From the point of view of qualitative analysis the resolution of this system was checked and compared with that of the original EDAX instrument, and a method is described how to determine the distribution of elements of the sample/possibly simultaneously/ by the aid of the scanning attachment of the microscope.

An independent mass measuring method /based on the Beer-Lambert law/ is presented which accomplishes the quantitative results obtained by the conventional microanalytical examinations. The mass measurements can be performed during the X-ray analysis. The reproducibility of electron optical parameters affecting the mass measurements was checked, and a restricted range of parameter values was given, in which a single mass thickness-thickness calibration curve characterises the system. A typical calibration curve is presented.

Several biological applications illustrating the efficiency of our assembly are shown and the mass change of the sample during the local excitation is demonstrated.

A MATHEMATICAL MODEL OF THE INTERACTION OF BACTERIUM_PHAGE COMPLEXES

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Our present model is an improved version of the mathematical description of the phage development cycle, developed earlier in the Institute of Biophysics. The former one was constructed for the interpretation of the "one-step" experiments. In those experiments we measured the number of phages by counting the plaques. According to the model the parameters characteristic of the phage development cycle are as follows:

c - mean value of the burst size

T - expected value of the burst size

σ - standard deviation of T

μ - adsorption rate constant.

The new model serves for interpretation of data obtained from optical measurements. This method enabled the construction of an automatized measuring system. While in the former case we measured the active phage contentration, in the latter one we counted the number of the non-lizated bacteria.

Starting points in constructing the model were as follows:

1. The phages are adsorbed with the same probability on infected and intact bacteria.
2. The lysis follows a normal distribution.

The following parameters can be determined from the model: T, σ , m and c /indirectly/, where m is the multipliation index, i.e. the ratio of number of phages to bacteria at the start of the process.

AUTOMATIZED MEASURING SYSTEM FOR STUDY OF BACTERIUM - PHAGE COMPLEXES

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The structure and biological function of bacteriophages are well-known, but their practical application /in biotechnology/ is yet scarce. Based on our experimental results and the mathematical model constructed for their interpretation a method has been developed for determination of the parameters characterizing the phage-development cycle: adsorption rate constant, latency period and its standard deviation, averaged burst size and multiplicity. An automatized measuring apparatus was constructed to ensure the reproducibility and reliability of the fast measurement of these parameters.

The programmable apparatus is suitable to carry out the following tests:

- Inactivation test. The phages in solution are exposed to well-defined physical and chemical agents. The biological consequences of the structural damage produced /e.g. the decrease of biological activity/ are determined quantitatively
- Cell-virus test. The parameter values of the phage development cycle depend on the biological state of the host cells. Thus, in a suitably-chosen bacterium-phage complex, the response to a given treatment of bacteria may model some speical cell--virus interaction processes.
- Phage-program test. During the measurement of the phage--development cycle parameters a simultaneous phage-

-development cycle takes place in the culture. The consequences of well-defined effects influencing definite parts of the phage-producing program /e.g. the effect of photosensitizers activated in a given time of the development cycle for a given duration/ are measured as changes of the parameter values.

STUDY ON SEGREGATION OF BACTERIA WITH PLASMIDS

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Based on the experimental results of segregation kinetics of bacteria with plasmids a theoretical model has been proposed which is in a good agreement with the experimental results and involves arbitrariness as small as possible.

The models involving different, but concrate regulations and stochastical elements were tested by computer simulation. Based on these experiences a general theoretical model was developed.

The most important assumptions of this model are as follows: 1. only one type of plasmids is present in bacteria. 2. the number of plasmid copies has a distribution and 3. the change in time of copy number is described by a homogenous Markov-process.

These assumptions can be fulfilled-according to our experiences in a chemostat. After studying the assimptotic behaviour of the above-mentioned Markov-process we have established that the stability of inheritance, the uniform segregation and the transition from a longer-lasting stable state to the uniform segregation state can also be described by means of this model. It also follows from the model that the distribution of plasmids during the process of segregation gets into an eigen-state, i.e. the mean value of copy-number per cell is constant with respect to plasmid containing cells.

According to our experience these theoretical results are in a good agreement with the experimental results.

COMPARISON OF MEASURED AND CALCULATED DOSE DISTRIBUTIONS

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A Honeywell-Bull 66/60 computer is used for planning radiation treatment of various tumours. Dose distributions are calculated according to the Van de Geijn program. This program takes into account the patient's cros-section with inhomogeneities in it and other data of treatment /size of fields, directions, places, etc./.

We examined whether the calculated curves would correspond to the measured ones in the following cases:

- isodose curves
- modification of dose distribution by surface
- modification of dose distribution by inhomogeneities
- modification of dose distribution by lead block
- modification of dose distribution by weight factor

After examining these questions found dose distributions to be correct with an acceptable uncertainty.

EFFECT OF BETA RADIATION ON THE ION TRANSPORT OF SMOOTH MUSCLE

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The effect of different dosages of beta radiation on Na and K content as well as on Na and K transport of smooth muscle of the small intestine was investigated. Isolated ilea of the frog were irradiated from equal distances and for different times with ^{90}Sr beta radiation source in normal Ringer solution containing ^{24}Na and ^{42}K. Non-irradiated preparations treated under the same conditions served as control.

During the experiments the smooth muscle preparations exhibited spontaneous peristalsis. The quantity of Na and K was determined after irradiation with 30, 60 and 120 Gy respectively. K and Na contents as well as specific activities were calculated for unit tissue weight. The results were evaluated on the basis of nearly 200 experiments. The Na and K content of the smooth muscle did not change upon the effect of irradiation. The specific activity of Na decreased after 30 Gy; at higher dosages it did not differ significantly from the control. Specific activity of K significantly decreased after 30 Gy, it did not change at 60 Gy and increased at 120 Gy. The K content of smooth muscle was 1/3 of that of the cross-striated muscle, but its radio-sensitivity was higher than that of the cross-striated muscle. According to our data the smooth muscle dos not differ from the sceletal muscle as far as the Na content and Na transport are concerned.

CONNECTIONS BETWEEN THE BIOLOGICAL EFFECT OF IONIZING RADIATIONS AND THE INDUCED LIGHT PHENOMENA

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As proved recently part of damages caused by ionizing radiation bacteria could be photoreactivated /Myasnik-Morozov, 1977/, similarly to the UV radiation damages. According to detailed investigations the greater part of these damages is produced by the mediation of Cerenkov-/UV/ light, evoked by the primary radiation /Michael-Harrop-Held, 1980/. But the smaller part of them can be detected also at low radiation energies, where the Cerenkov-effect is not yet standing out /Moss-Smith, 1980/. This smaller part is probably due to the direct excitation of biomolecules /Smith, 1976/.

On the basis of our experiments performed on cross-striated muscles we suggested in 1975 that the radio-luminescence evoked by tritium beta radiation of low energy, probably due to direct excitation, could also have a mediator role in a the radiation effect of complex mechanism: in the decrease of stimulus threshol caused by the same radiation. We proved that pyridoxin and tryptophan which increase the radioluminescence intensity would facilitate the stimulus-threshold decreasing effect of incorporated tritium. In the experiments presented here we obtained similar results when the muscles were irradiated with an external high-energy beta source or with X-ray of energy below the Cerenkov threshold.

EFFECT OF TRITIUM BETA IRRADIATION ON THE PLASMA MEMBRANES OF CULTURED CELLS. I. ALTERATIONS IN RECEPTOR FUNCTIONS

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The effects of tritium beta irradiation on plasma m membranes of various cultured cells were studied in long- and short-term experiments. A concentration of 3.7 10^{-2} kBq per ml of either ^{3}H-thymidine or ^{3}H-water /HTO/ did not influence the concanavalin A binding capacity of CHO cells exposed in long-term experiments. 3.7 kBq per ml in either form, however, caused well-detectable alterations in the lectin-binding. The release of cells from tritium irradiation by replacing them into tritium-free medium resulted in rapid restoration of surface conditions. The surface alterations can be attributed to the direct effect of low energy beta irradiation on the plasma membranes.

Short-term experiments were performed at a concentration of 37 kBq per ml of HTO on the plasma membranes of primary human embryonic fibroblasts and primary green monkey kidney cells. Tested were the receptor activities for concanavalin A and polio 2 type virus. The amount of bound concanavalin A did not change one minute after HTO exposure. Exposure for ten minutes, however, caused a considerable, 1.5 to 2 fold increase above the control value, while strong depressed values were found after a 60 min exposure. The development of cytopathogenic effect following infection of cells with polio 2 virus was

delayed in HTO-pretreated cells. The effect depended on the duration of virus-cell contact. Five minutes adsorbtion time proved to be not enough to cause successful infection on the radiation-perturbed plasma membrane. From experiments comparing the effects of D_2O /0.1-20%/ and HTO it can be concluded that the beta irradiation, but not the mass effect, is responsible for the surface alterations as D_2O did not produce any changes detectable by our sensitive technique.

EFFECT OF TRITIUM BETA IRRADIATION ON THE PLASMA MEMBRANES OF CULTURED CELLS. II. MORPHOLOGICAL APPEARANCE

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Ultrastructural and morphological alterations of primary green monkey kidney cells were studied by electronmicroscopic techniques after treatment of the cells with 37 kBq per ml tritiated water /HTO/. The results indicate that upon the effect of HTO vacuolization of endoplasmatic reticulum, filamentations of cells occurred; the cells were thinned, their surfaces became smooth, and a stronger contact of cells to substrate developed. In contrary, a much higher concentration of deuterium /10%/ than that of the applied tritium did not cause similar micromorphological alterations.

It was also observed by the use of polycationized ferritin that the quantity and topology of negative surface charges on cells had been changed upon the effect of low energy beta irradiation, i.e. the areas having negative charges as well as the density of charges on cell surfaces had decreased considerably.

EFFECTS AND DOSIMETRY OF MICROWAVE /2450 MHz/ IRRADIATION ON CHICK EMBRYOS AND RATS

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The study of potentially hazardous microwave radiations is of utmost importance. The exposure limits for microwaves are different in the various national standards, therefore, the investigations of the complexicity of biological effects of microwave fields is of special interest.

In our experiments we applied different dose-rates /between 0.1 mW/cm^2 and 2 W/cm^2/ at a frequency of 2450$\pm$ 50 MHz, C.W. in cavity resonator system and in free space, respectively.

The experiments were performed on white Leghorn chick embryos of different ages /incubations: 37.5$\pm$0.5°C, 70% humidity/ and on 180 g F_1 hybride male rates.

The specific absorption rate /SAR/ was determined in chick embryos. Irradiations of the embryos on the 15th day of incubation were performed at a SAR value of 35 mW/g. Biochemical investigations were made on day 19 of embryogenesis. The brains and livers of control, i.e. non-irradiated and microwave--irradiated chick embryos were removed after decapitation, collected, cooled and homogenized. Components of protein-

biosynthesis were isolated from brain and liver homogenates. The protein synthesizing function was measured *in vitro*. Significant post-irradiation differences were found in the brain.

Furthermore, we studied the effect of low power rate /0.1-100 mW/cm^2/ on the physiological functions of the rat brain. EEG records were taken before, during and after irradiation. Evaluation of the EEG patterns revealed transient changes at low power rates.

Comparison of the experiments *in vivo* and *in vitro* are in progress.

References:

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RADIOSENSITIZATION OF BACILLUS MEGATERIUM SPORES BY CARBON DIOXIE

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The radiosensitivity of Bacillus megaterium spores in carbon dioxide was studied at four different oxygen concentrations /100% CO_2, 0.8%, 1.6%, 4.2% and 7.9% O_2 in CO_2/ Seven oxygen concentrations in N_2 were used as control/ 100% N_2, 0.7%, 1.2%, 2.1%, 3.1%, 5.1% 21% and 100% O_2/ No radiosensitizin action was observed in spores in suspension equilibrated with 100% CO_2 or 0.8% O_2 in CO_2. Further increase in O_2 concentration /1.6% O_2 or 4.2% O_2 in CO_2/ enhanced the lethal damage when compared with oxygen-in-nitrogen gas mixture. On spores equilibrated at 7.9% O_2 in CO_2 no radiosensitizing effect was exerted.

Further radiochemical experiments are needed for a better understanding of how does CO_2 sensitize the spore system.

EFFECT OF 0.5 Gy IN UTERO NEUTRON IRRADIATION ON MOUSE EMBRYOS

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Female C57Bl/6 mice were exposed to single dose of 0.5 Gy fission neutrons on day 18 of pregnancy. Postnatal lethality increased from the control 5 % /6/118/ to 27.6 % /32/116/ in irradiated mice within the first 3 days after birth. The 30-day postnatal survival data showed that the first 72-hour neonatal period is very critical. At age of 32 days the body weight of irradiated animals was 30 % lower than that of the controls. The brain weights of 21-day-old animals, irradiated in utero, were under the control; this was not due to the water content of the brains. In our earlier work it was shown that the RNA and DNA content /related to wet tissue weight/ and the incorporation of precursors into DNA and nuclear RNA did not change significantly in the brain, but the protein content slightly decreased. Incorporation of ^{14}C-amino acid mixture into acid-soluble nuclear proteins diminished by 40 %. The amount of H4 histone markedly decreased in the brain. Now, we found that the incorporation of ^{35}S-methionine into certain non-histone protein fraction decreased after irradiation. These proteins were separated by two-dimensional electrophoresis according to O'Farrell. Histological examinations of embryonal brain were performed 6 hours

after the in utero irradiation. Damaged areas were seen on the serial sagittal sections near the ventricles. Especially high number of damaged cells were found in the olfactori areas, cerebral cortex and corpus callosum. Near the third ventricle even about 50 % of cells were damaged. The external granular layer of the cerebellum was also markedly injured.

According to the authors' hypothesis neuroblasts are very sensitive to radiation.

CHANGE OF SUPEROXYDE-DISMUTASE /SOD/ ENZYME ACTIVITY UPON THE EFFECT OF VARIOUS OXYGEN CONCENTRATIONS AND IONIZING RADIATION

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The superoxyde-dismutase enzyme /E.C. 1.15.1.1./ belonging to the class of oxydo-reductases can be found in any aerobic organ. During oxygen metabolization it affects the dismutation and elimination of superoxyde anion /O_2^-/ formed in the course of one or two-electronic reduction of the oxygen molecule.

Studied were the changes of enzyme activity <u>in vitro</u> upon the effect of various oxygen concentrations and increasing doses of irradiation. The measurements were performed by the method based on inhibition of adrenaline auto-oxidation described by Mishra and Fridovich /1972/.

SOD (220 /ug/ml) was dissolved in distilled water and enzyme activity determined in solutions bubbled, with 100% N_2, 100% O_2, 21% O_2 in N_2 gas mixture, and 3.1% O_2 in N_2 gas mixture. Significant difference was found in the N_2 gas saturated system. Furthermore, solutions were irradiated in PX-gamma30 apparatus and the alterations in enzyme activity of the samples measured. Significant differences in activity decrease were observed in solutions of low oxygen concentration.

EFFECT OF SELENIUM COMPOUNDS ON BIOLOGICAL SYSTEMS

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The present publication deals with some results and perspectives of investigations on the effect of selenium on biological systems.

Experiments will be described where the application of selenium-containing compounds has been aimed for radiation protection or against cancer, because of the high biological activity of selenium compounds, among others of their antioxidant properties.

In connection with the possible efficacious medical application in biological membranes suggestions are made for selenium treatment of pathological conditions related to intesification of oxidant reactions.

ROLE OF R 46 /pkM 101/ PLASMID IN RADIORESISTANCE

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R 46 /pkM 101/ was transferred to wild-type E.coli K 12 AB 1157 and to its rec mutants /rec A 13, rec B 21, rec C 22, rec F 143/ by conjugation. The apperence of R 46 /pkM 101/ R factor in rec^+ rec^- E.coli strains was detected by conferring resistance to ampicillin.

After Co-gamma irradiation R 46 /pkM 101/ slightly increased the survival of the wild-type strain.

The survival curves of R^+ and R^- rec mutant strains were different.

It seems that /pkM 101/ is able partlially to compensate for the lack of encoding activities of gene products of certain mutants.

LASER-SPECIFIC BIOSTIMULATION: REALITY OF MYTH?

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Every time when a new sort of energy source is discovered, people interested in healing hope to have found the "philosopher's stone". This happened after X-rays were discovered, this was the case with ultrasound, and this is the ituation at present with the lasers, too, proliferated by the old desire to heal with light. Beside the practice of using lasers for surgical purposes, i.e. using the high energy density available in focused laser beams for healing by destroying, reports on alteration of biological systems upon irradiation with low intensity lasers, i.e. with intensities not causing denaturation of the biomolecules, rose considerable interest and enthusiasm. Claims have been made on the existence of laser-specific biostimulation.

The scope of this presentation is to analyse in the light of biological photochemistry whether these reported phenomena which range from wound healing to laser acupuncture are laser -specific indeed or reflect the fact that these biomedical applications were rendered possible only after lasers as light sources of efficient brightness had become available and that the laser interaction with the living material could at least in principle be induced by thermal sources as well.

For a photochemical reaction it is typical that it takes place from an usually electronically excited state of the molecule that absorbed the photon or to which the energy was

transferred in an identifiable non-statistic manner. Most of transferred in an indentifiable non-statistic manner. Most of induced by UV radiation only, preferably at wavelengths below 320 nm, in the IR, but practically not in the visible spectrum in which the claimed laser-specific biostimulation effects have been described.

We wish to demonstrate theoretically and experimentally that the phenomena described so far cannot be attributed only to a specific laser-effect, they can be reproduced by other means too.

EFFECT OF LASER IRRADIATION ON THE PROSTAGLANDIN RECEPTOR OF ERYTHROCYTES AND ON THE PROTEIN SYNTHESIS OF EPITHELIUM CERVICIS UTERI

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Low-dose fractionated He-Ne laser irradiation can successfully be applied for the treatment of ectopium cervicis uteri. The favourable therapeutical effect is considered as the result of complex biological processes. In order to clarify the biochemical mode of action two-directional model-experiments were performed in vitro;

a/ changes occurring in the functional state of cell membrane were determined by measuring the prostaglandin E_2 binding ability of human erythrocytes;

b/ to demonstrate stimulatory or inhibitory effects in cell metabolism, the intensity of protein biosynthesis was measured in human portio uteri tissue slices, obtained from surgical interventions, after separating parts covered with pavement epithelium and cylindrical epithelium.

The binding ability of PGE_2 receptors on the surface of erythrocytes changed to a small extent upon the effect of He-Ne laser irradiation /λ = 632.8 nm, 2.41 mW/cm^2 output, 0.3-9 J/cm^2 energy/. Irradiation with nitrogen dye laser at various wavelengths showed significant deviation. At 585 nm, 1-2 J/cm^2 energy caused abt. 40% binding-increase, at 465 nm dose dependent decrease was observed; changes were of transient and reversible character

since 18 hr after irradiation the PGE_2 binding ability of erythrocytes was restored to normal.

The protein synthesis of portio uteri slices irradiated with 5-20 J/cm^2 He-Ne laser changed in a different manner in parts covered with cylindical and pavement epithelium. While the amino acid incorporation of the cylindrical epithelium significantly decreased after irradiation, the incorporation of pavement epithelium slightly increased. Similar tendency, though less differences appeared also in labelling of the intracellular amino acid pool. This indicates that the transport of amino acids into the cells was altered.

Consequently, He-Ne laser radiation induces inhibitory and stimulatory effects on protein synthesis of portio epithelium depending on the type of tissue. This selective effect can directly be correlated with the beneficial therapeutical results obtained with laser treatment, namely that He-Ne irradiation applied on ectopium cervicis represses the growth of cylindrical epithelium, stimulating simultaneously the vaginal pavement epithelium proliferation restoring herewith the normal tissue equilibrium.

EFFECT OF ACUTE ^{60}Co-GAMMA IRRADIATION ON LIPID PEROXIDATION /MALONALDEHYDE LEVEL/ IN RAT TISSUES

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The effect of sublethal /6.0 Gy/ and LD /9.0 Gy/ as well as of supralethal /12.0 Gy/ doses of acute ^{60}Co-gamma irradiation was studied on the development of malonaldehyde level /MDA/ in rats.

Acute ^{60}Co-gamma irradiation generally increased the formation of MDA as the main product of lipid-peroxidation in the organs studied /brain, liver, spleen, kidneys, testes, stomach, small intestine/ to an extent characteristic of the organs and as a function of time.. From the differences in the rate of change and time of occurrence conclusions can be drawn as to the radiosensitivity of the organs. Based on this one may suppose that the irradiation-induced increase in MDA-level contributes considerably to the development of some symptoms of radiation sickness.

INVESTIGATION INTO THE EFFICIENCY OF SPECIFIC TREATMENTS APPLIED SIMULTANEOUSLY IN CASE OF INTERNAL CONTAMINATION WITH RADIOIODINE AND RADIOCESIUM

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Based on investigations performed in the last few years, the toxicological properties, metabolism and the mechanism of elimination of some biologically hazardous radionuclides /$^{89-90}Sr$, ^{131}I, ^{137}Cs, etc./ might be considered as clarified. In the experiments aiming at prevention and therapy of internal contamination, primarily specific methods were used in order to decrease the retention and to increase the elimination of some radionuclides. However, data regarding the contamination with isotope-mixture and the efficiency of therapeutical treatments applied simultaneously are scarce enough.

Starting from the foregoing, experimental work was done to reveal the relations of deposition and elimination in rats with internal contamination caused by the mixture of radioiodine and radiocesium which nuclides occur among fission products in a significant amount, and to determine the responsiveness of the rate of elimination by adding decorporating compounds separately and in combination. ^{131}I and ^{134}Cs were used as labelling isotopes.

Results obtained from these experiments prove that the mobilization of the above-mentioned nuclides can beneficially be performed with the simultaneous, combined application of

specific agents like stable iodine, colloidal Prussian-blue without diminishing the efficiency found in the case of monotherapy.

EFFECT OF MIXED FISSION NEUTRON+GAMMA WHOLE-BODY IRRADIATION ON THE RETENTION OF ^{144}Ce IN MICE

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In the case of internal contamination with radioisotopes, also the effect of external irradiation /mainly gamma-ray and neutrons/ on the living organism should be taken into account, besides the incorporation of radionuclides into the organism. External radiation, even in relatively low dose, may cause functional and pathological changes.

Considering the significance of neutron irradiation as a potential possibility, it is of extreme importance to clarify the modifying effect of this radiation in radiotoxicology.

Starting from what was said above, our experimental work on mice aimed at investigating the effect of various doses /0.5; 1.0; 1.5 and 3.5 Gy/ of fission neutron+gamma whole-body irradiation on the organism exposed to an internal contamination with radiocerium /$^{144}CeCl_3$/, which can be found among fission products in a significant amount /appr. 4.6-6.0 %/. According to the experiments the deposition and the rate of elimination of radionuclides can advantageously be influenced by the combined administration of decorporating and radioprotecting compounds /DTPA; DFOA; AET/.

INVESTIGATION ON ACTIVITY CHANGES OF MONOAMINOOXIDASE IN THE BRAIN AND LIVER OF EXPERIMENTAL ANIMALS EXPOSED TO FISSION NEUTRON-GAMMA IRRADIATION

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The changes of the activity of monoaminoaxidase /MAO/, an enzyme playing a key role in the metabolism of neurotransmitters, were determined as a function of time after various ionizing radiations and radioprotector pretreatment in the brain and liver of experimental animals. MAO activity was found to decrease in the brain and simultaneously to increase in the liver after fission neutron-gamma irradiation. In contrary, cerebral MAO activity significantly increased following ^{60}Co-gamma irradiation, while the change in the liver was similar to the values found after fission neutron-gamma irradiation, i.e. the same increase could be demonstrated.

AET /S_2 - aminoethyl-isotiuronium Br.HBr given in a radio-protective dose inhibited MAO in the brain but decreased enzyme activity in the liver, when compared to the control. Following AET pretreatment and fission neutron-gamma irradiation an even more significant activity change was observed both in the liver and the brain.

From the results obtained we assume that different kinds of ionizing radiation affect lipidperoxidation in the lipids of microenvironmental of MAO, in different ways, an event resulting in unlike changes of enzyme activity AET which itself decreased MAO activity in the brain and increased it in the liver did not prevent the enzyme activity changes due to the effect of fission neutron-gamma irradiation.

COMPARISON OF THE EFFECT OF GAMMA AND 14 MeV NEUTRON IRRADIATION ON THE ENZYME ACTIVITIES OF PEA SEEDLINGS

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Seeds and four-day-old pea seedlings were irradiated with zero to 300 Gy doses of ^{60}Co gamma as well as 14 MeV neutron radiation. Growth inhibition and enzyme activity examinations were carried out on the one-week-old etiolated seedlings.

After seed irradiation the height of seedling decreased linearly with increasing gamma dose up to 300 Gy, while after seedling irradiation a rapid decrease between 0 and 30 Gy and only a slight decrease from 30 to 300 Gy were observed. Neutron irradiation of seeds was ten-times more effective in inhibiting growth than was gamma irradiation.

As reported earlier gamma irradiation of plants increased the activity of degradative enzymes /e.g. RNase, G. Bagi: Acta Biochim. Biophys. 15, 129/ but decreased that of some others /e.g. RNA polymerase/. Comparison of dose-response curves of peroxidase, RNase and nucleoside triphosphate diphosphatase enzyme activities after irradiation of seeds and 4-day-old seedlings revealed that after seed irradiation the enzyme activity increased in proportion to the dose up to 300 Gy, while after seedling irradiation the enzyme activities increased rapidly at low doses, reaching a maximum at 30-50 Gy, then

slowly decreased up to 300 Gy. There were close correlations between the dose-response curves of enzyme activity and the dose-response curves of growth inhibition: they reflected each other.

In the case of growth stimulation at low dose the enzyme activity decreased below the control level. After neutron irradiation the trend of enzyme activity change was the same, but neutron irradiation proved to be ten-times more effective than gamma irradiation.

NUCLEAR IMAGING WITH MOVING AND STATIONARY DETECTORS

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The paper discusses theoretical principles of nuclear imaging and localisation of distribution of activity. In the first phase of technical development scanning procedures were elaborated that were suitable for imaging quasi-stationary distributions. The accuracy, quality and transmission characteristics of imaging are discussed here. Problems of visualisation are dealt with in details. Questions of secondary visualisation improving unfavourable characteristics of direct visualisation are discussed.

Possible methods of simultaneous imaging with stationary detector are presented followed by the discussion of the spark chamber. Scintillation cameras, their principle of operation, transmission characteristics, quality parameters and methods of visualisation are then discussed in details. The use of on-line computers and data processing make full expoloitation of scintillation cameras possible. Practical tasks and methods characterizing modern nuclear imaging are then discussed in details. The present paper does not deal with the latest development in imaging, i.e. emission computer tomography. Questions related to this topic will be dealt with in another paper.

QUALITY CONTROL SYSTEM OF GAMMA CAMERAS

Sára Keszthelyi - Lándori

Gamma Works, Budapest

Interpretation of performance parameters /uniformity, linearity, spatial- and energy resolution, countrate capability, sensitivity/.

Measuring methods for camera detector and for complete camera systems.

Comparison of PICKER, GAMMA and NEMA specification standards. Practical questions.

EMISSION COMPUTED TOMOGRAPHY /ECT/, NEWER TENDENCIES, IN NUCLEAR IMAGING

L. Csernay

Department of Nuclear Medicine, Medical University, Szeged

Two fundamentally different types of computed tomography are known the transmission and emission ones. Both methods are based on mathematical methods of three-dimensional image reconstruction techniques. At the ECT it is a complicating factor that correction for tissue absortion should be taken into account. The report deals with the possible solution forms of ECT and experiences of clinical examinations abroad upto now. The author reviews in detail the results of simulation studies in the Department of Nuclear Medicine of the University Medical School of Szeged and the problems of technical and software development of a prospective emission computer tomograph made in Hungary.

Recently beside ECT the positron ECT has obtained more and more scientific importance. The PECT detects the distribution of short-lifetime positron-emitting cyclotron products within the organs and the dynamic change of this distribution. The report shortly summarizes the used technical solutions and the most important clinical results. Finally the author deals with the expectable perspectives of nuclear imaging methods.

RADIOPHARMACEUTICALS IN THE MODERN NUCLEAR MEDICINE

J. Láng

Department of Nuclear Medicine, Medical University, Szeged

Recently the position of the users of radiopharmaceuticals is simpler then 10-20 years ago when only centrally produced radiopharmaceuticals existed. These products were delivered into the laboratories only in defined times. Nowadays the radiopharmaceuticals obtained from widely used various local generator systems /^{99}Mo-^{99m}Tc, ^{113}Sn-^{113m}In, ^{81}Rb-^{81}Kr/, ensure great independency in time for the users. The central delivery is limited besides the generators only to cyclotron products of medium half-life /^{67}Ga, ^{111}In, ^{123}I, ^{201}Tl/.

The paper deals with:

1/ the most frequently used generator system /^{99}Mo-^{99m}Tc/, the production of radiopharmaceuticals labelled with ^{99m}Tc, their quality control and the most important aspects of their use;

2/ organization problems of the cyclotron product radiopharmaceuticals which can be used far-off the cyclotrons;

3/ prospective products of the Hungarian cyclotron and organization problems in connection with their use;

4/ the problems /labelling, synthesis, quality control/ relating to the use of positron-emitting radionuclids /^{15}O, ^{13}N, ^{11}C/.

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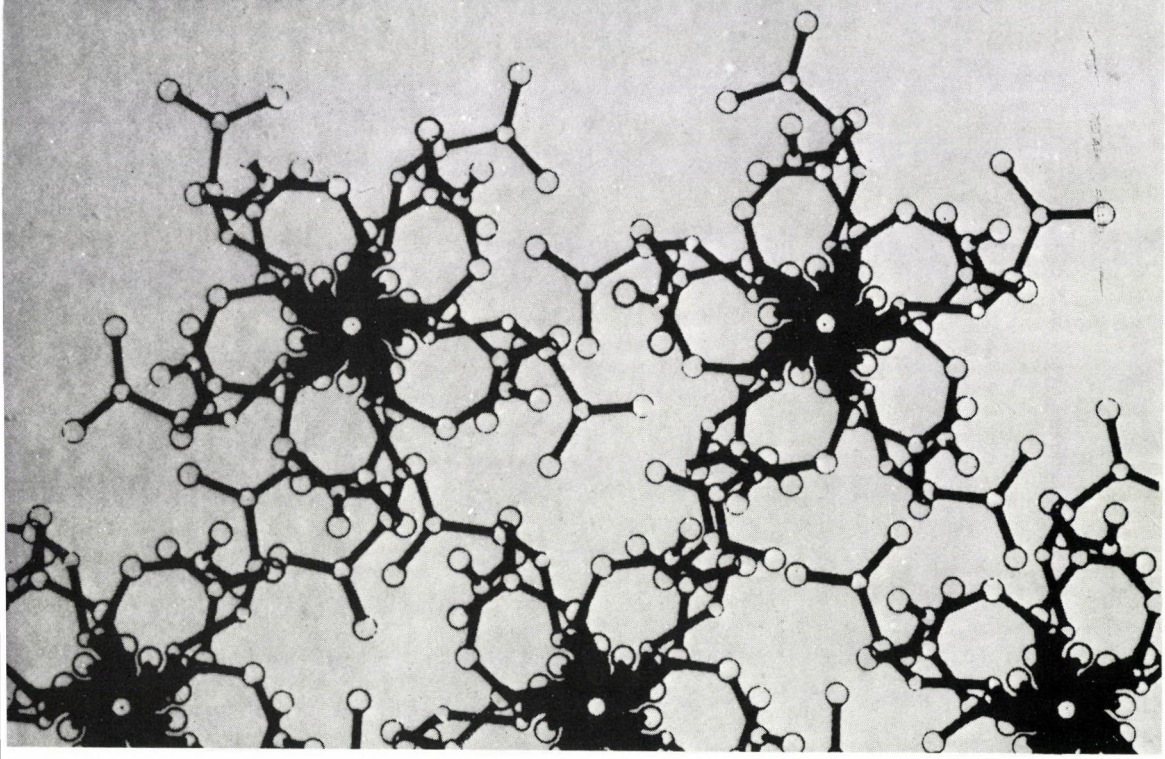

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 18 (3—4), pp. 125—149 (1983)

The Present State of the Chemiosmotic Coupling Theory*

H. V. WESTERHOFF,[1] S. L. HELGERSON,[2] S. M. THEG,[3] O. VAN KOOTEN,[4] M. WIKSTRÖM,[5] V. P. SKULACHEV,[6] Zs. DANCSHÁZY[7]

(Received February 15, 1983)

Although the general principles of the chemiosmotic coupling theory have become widely accepted, the (degree of) loc(aliz)ation of electrochemical proton potential difference cannot yet be deduced from the existing experimental data. Many results are not in ready accordance with the idea that one protonic electrochemical potential difference, i.e. the one between a homogeneous inner and a homogeneous outer aqueous phase, would be the high-free-energy intermediate of membrane-linked free-energy transduction. Rather, free-energy transduction in an organelle like a mitochondrion or a chloroplast might take place in large number (about 1 per $\vec{H}^+$-ATPase) of miniature chemiosmotic systems. The energized protons produced in such a miniature system might be largely (but not totally) confined to a proton-domain belonging to it. Hence, there might be *many* (rather than one) different relevant proton gradients.

Structure

I. Survey of the literature on cases where delocalized chemiosmotic coupling seems to fail

II. Some additional recent data

* A summary of the round table discussion held September 1982, at the ICRO-UNESCO training course on "Electrogenic Effects in Bioenergetics", Szeged, Hungary. Authorship does not imply consent with everything written, except in sections with the authors name.

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Abbrevations: A23187, a Ca^{2+} ionophore; Chl, Chlorophyll; DBMIB, 2,5-dibromo-3-methyl-6-isoprophyl-p-benzoquinone; DCCD, dicyclohexylcarbodimide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; CCCP, carbonyl cyanide-m-chloro-phenylhydrazone; DMO, 5,5-dimethyloxazolidine-2,4-dione; DMSO, dimethyl sulphoxide; E. C., energy charge = = (2[ATP] + [ADP])/([ATP] + [ADP] + [AMP]); FCCP, carbonyl cyanide p-trifluoromethoxy phenyl hydrazone; ΔG_0, Gibbs free energy of redox reaction; ΔG_p, Gibbs free energy of ATP hydrolysis reaction; HEPES, N-1-hydroxyethylpiperazine-N'-2-ethane sulfonic acid; J_0, oxidation rate; J_p, phosphorylation rate; L_0, activity of the respiratory chain; L_p, activity of the $\vec{H}^+$-ATPase; MOPS, morpholinoethane sulfonic acid; PMS, phenazine methosulphate; $TPMP^+$, triphenyl methyl phosphonium ion; TPT, triphenyl tin; ΔpH, pH gradient across the membrane; $\Delta\Psi$, electric potential difference across the membrane; $\Delta\tilde{\lambda}_H$, local electrochemical potential difference for protons; $\Delta\tilde{\mu}_H$, delocalized electrochemical potential difference for protons, i.e. from bulk phase to bulk phase.

III. Criticism on I and II: defending delocalized chemiosmotic coupling
IV. Alternatives to delocalized chemiosmotic coupling
V. Conclusions
VI. The proton cycle and the local field

I. Survey of the literature (by H. V. Westerhoff)

In his 1961-Nature and his later papers on chemiosmotic coupling Mitchell (1961, 1979) proposed and elaborated what can be divided into two hypotheses. The one we shall discuss here (i.e. the physiological level chemiosmotic coupling theory; Mitchell, 1981), proposes that a proton gradient across the mitochondrial (or: energy coupling) membrane functions as the high energy intermediate in oxidative phosphorylation. The other (set of) hypothesis proposes specific, redox loop, mechanisms for pumps to operate. Although reduction of cytochrome c by ubiquionol ('site 2') is probably catalyzed by a redox loop, other proton pumping mechanisms are probably (also) operative in the cases of cytochrome oxidase (Wikström, Krab, 1980) and bacterorhodopsin (Stoeckenius, Bogomolni, 1982).

In Fig. 1 we summarize the principles of the chemiosmotic coupling theory. Two aqueous phases are separated from each other by a membrane that is almost impermeable to H^+ and OH^-: the only ways for protons to leak from one aqueous phase to the other is through the proton-leak or through the $\vec{H}^+$-ATPase (see below). When catalyzing the reduction of oxygen by NAD(P)H, reduced flavoprotein, or cytochrome c, the respiratory chain pumps a (substrate-specific) number of protons from the one aqueous phase (the 'N-domain') to the other (the 'P-domain'). This will acidify the P-domain and alkalizine the N-domain, but, because protons carry a positive charge, it will also generate a transmembrane voltage ('electrical potential') difference (compare this to the transport of an electron from one plate of a condenser to the other). Both the pH gradient (ΔpH) and the transmembrane electrical potential ($\Delta\Psi$) will tend to push protons backward. The sum of these pushing tendencies is the Gibbs free energy difference between a proton in the P-domain and a proton in the N-domain: the 'transmem-

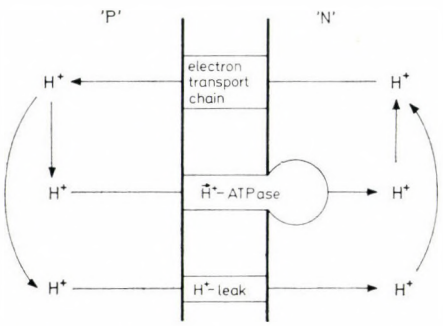

Fig. 1. The "physiological level" chemiosmotic coupling scheme proposed by Mitchell (1961). (P: positive, acidic side; N: negative, alkaline side)

brane electrochemical potential difference for protons ($\Delta\tilde{\mu}_H$)'. At room temperature:

$$\Delta\tilde{\mu}_H \text{ (in kJ/mol)} = -5.7 \cdot \Delta\text{pH(in units)} + 0.096 \cdot \Delta\Psi \text{ (in mV)}.$$

The $\vec{H}^+$-ATPase catalyzes the reaction:

$$n\ H_P^+ + ADP_N + P_{iN} \rightleftarrows (n - n_S)H_N^+ + ATP_N$$

Here n_s, the number of protons involved in the ATPase reaction as it would occur in the absence of a membrane (about 0.5 at pH 7), is irrelevant for the free-energy transduction. The subscripts P and N refer to the two respective domains.

When the difference in electrochemical potential between H_P^+ and H_N^+ (i.e. $\Delta\tilde{\mu}_{H,P-N}$) becomes sufficiently high, i.e. higher than the Gibbs free-energy of ATP hydrolysis ($\Delta G_P = 50 + 5.7 \cdot \log[ATP]/([ADP] \cdot [P_i])$ kJ/mol, if concentrations are expressed in mM-total) divided by n, this reaction will start running to the right, i.e. in the direction of ATP synthesis (cf. Mitchell, 1961, 1979, Westerhoff et al., 1981b). ATP synthesis coupled to respiration will then occur. In photosynthetic systems photo-redox chains or bacteriorhodopsin would play the part of the respiratory chain. The membrane would be the thylakoid membrane of chloroplasts, the plasma membrane of bacteria, or the inner mitochondrial membrane of eukaryotes.

A remaining problem is the identity of the P- and N-domains. In view of the rapid diffusion of protons in aqueous media (Mitchell, 1979), Mitchell suggested that the mitochondrial matrix space and the space outside the inner mitochondrial membrane would correspond to the N- and P-domains, respectively, each of which would be homogeneous with respect to the electrochemical potential for protons. Thus, although on their way from the electron transport chain to the $\vec{H}^+$-ATPase the functional protons might preferentially take routes close to the membrane (Mitchell, 1979, 1981) their Gibbs free-energy would be essentially delocalized all over the aqueous phase on that sideof the membrane. The relevant $\Delta\tilde{\mu}_H$ would be the proton 'selectrochelmica potential difference between the two bulk aqueous phases. We shall name this point of view 'the delocalized chemiosmotic coupling theory'.

Around 1977 (Mitchell, 1981) many bioenergeticists agreed that, to say the least, the delocalized chemiosmotic theory was the best working hypothesis for energy coupling. The most convincing experiments had shown that (review: Boyer et al. 1977): (a) electron chains and bacteriorhodopsin can give rise to proton movement, ΔpH and $\Delta\Psi$ (transmembrane electric potential difference) as measured with bulk-bulk phase probes; (b) the same is true for the ATPase; (c) imposed ΔpH and $\Delta\Psi$ can drive ATP synthesis; (d)liposomes plus (semi ?) pure F_0F_1 and pure bacteriorhodopsin catalyse light-driven ATP synthesis; (e) uncouplers have protonophoric (i.e. proton translocating) activity.

For us the conclusion must be that the bulk phase transmembrane difference in electro chemical potential ($\Delta\tilde{\mu}_H = Z\Delta\text{pH} + F\Delta\Psi$) of protons is rather closely related, ifnot identical, to the high-energy intermediate of energy coupling. All alternative coupling schemes will have to take this and the above experimental results into account. On the other hand, these experiments do not constitute defin-

itive proof that $\Delta\tilde{\mu}_H$ is the (only) high energy intermediate. The alternatives to delocalized chemiosmotic coupling (Fig. 2A) given in Figs 2B and 2C are equally consistent with these results.

From the delocalized chemiosmotic coupling scheme (cf. Fig. 2A Fig. 21) the following consequences can be derived or suggested:

(i) The redox reaction rate (J_0) should depend on ΔG_p (i.e. ADP, ATP and phosphate concentration) only through changes in $\Delta\tilde{\mu}_H$, i.e. $J_0 = J_0(L_0, \Delta G_0, \Delta\tilde{\mu}_H)$ in which L_0 represents the catalytic activity of the redox chain.

(ii) Similarly the phosphorylation rate $J_p = J_p$ (L_p, ΔG_p, $\Delta\tilde{\mu}_H$), in which L_p represents the catalytic activity of the H^+-ATPase.

(iii) At 'state 4' or 'static head' of ATP synthesis, (when the $\vec{H}^+$-ATPase reaction itself has come to equilibrium) $\Delta G_p/\Delta\tilde{\mu}_H$ should be constant, i.e. independent of $\Delta\tilde{\mu}_H$, equal to the $\vec{H}^+$-ATP stoichiometry of the $\vec{H}^+$-ATPase. (cf. Westerhoff et al., 1981b). This argument is insensitive to allosteric interactions between electron transport chain and $\vec{H}^+$-ATPase.

(iv) Since energy coupling organelles contain large numbers of proton pumps, which share one $\Delta\tilde{\mu}_H$, the latter is expected to have 'pool-properties'. Thus partial inhibition of either of the two proton pumps in Figs 2A and 3 (i.e. the redox proton pump, or the $\vec{H}^+$-ATPase) is expected to make the other less limiting in the overall reaction (Baum et al., 1971; Hitchens, Kell, 1982a; Westerhoff et al., 1983).

(v) Protonophoric uncouplers should be able to shuttle across the membrane.

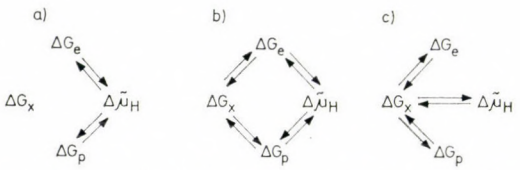

Fig. 2. Energetic representations of (a) the macroscopic chemiosmotic coupling scheme (b) the parallel coupling scheme (Padan, Rottenberg, 1973) and (c) 'forked' type coupling schemes (cf. Westerhoff et al., 1981b). ΔG_e (e.g. $\Delta G_e^{0\prime} + RT \cdot \ln([NADH] \cdot pO_2/[NAD^+])$, ΔG_p ($\Delta G_p^{0\prime} + R \cdot T \cdot \ln([ATP]/([ADP] \cdot [P_i]))$ represent the redox potential difference of the substrate and product couples of the redox reaction and the phosphate potential (= free energy of hydrolysis per mole of ATP). J_0, J_p and J_H^l represent the redox reaction rate, the rate of ATP synthesis and the rate of (passive) proton back leakage across the membrane

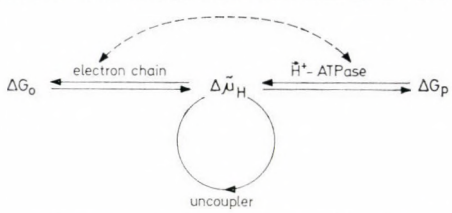

Fig. 3. The delocalized chemiosmotic coupling scheme with allosteric interaction superimposed (dotted line)

(vi) After inhibition of the energy consuming proton pump (i.e. $\vec{H}^+$-ATPase in oxidative phosphorylation or the respiratory chain in reversed oxidative phosphorylation ('reversal')) more protonophore should be needed to decrease $\Delta\tilde{\mu}_H$ to the extent that the energy consuming (uphill) reaction, (e.g. ATP synthesis or NAD^+ reduction by succinate) can no longer occur.

These consequences of the delocalized chemiosmotic coupling scheme have been tested and found to be absent: (e.g.)

Ad. i: Padan and Rottenberg (1973) added ADP or varying amounts of protonophore to mitochondria at identical ΔG_0. They measured respiratory rate J_0 and $\Delta\tilde{\mu}_H$ and found that with ADP the respiratory rate was much higher than with the amount of protonophore that lowered $\Delta\tilde{\mu}_H$ to the same value (Fig. 4).

Comments. (M. Wikström): In these experiments $\Delta\tilde{\mu}_H$ was measured with centrifugation. Thus anaerobiosis may have occurred with concomitant leakage of the $\Delta\tilde{\mu}_H$-probes out of the mitochondria, more so under conditions with protonophore than under conditions with ADP.

(H. V. Westerhoff): In these experiments $\Delta\Psi$, the major component of $\Delta\tilde{\mu}_H$ was strongly 'buffered' by the considerable amount of K^+ being present on

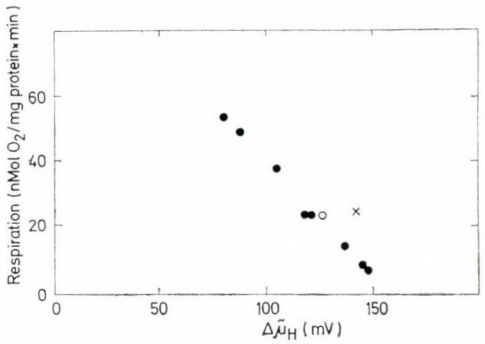

Fig. 4. The relation between the respiration rate and $\Delta\tilde{\mu}_H$ in rat liver mitochondria. (●) Valinomycin + KCl; (○) dinitrophenol; (*) phosphorylation. (From Padan and Rottenberg, 1973, reproduced with kind permission of Dr. Rottenberg and FEBS)

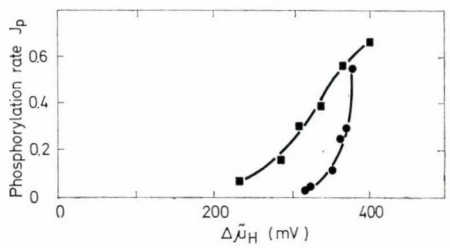

Fig. 5. The relationship between the rate of phosphorylation (in mmol · h^{-1} · mg $BChl^{-1}$) and $\Delta\tilde{\mu}_H$ under conditions of partial uncoupling and limiting light intensity in highly active chromatophores. The concentration of FCCP varied from 1×10^{-1} to 2×10^{-6} M(■). The intensity of the incident actinic light was changed from 1.2×10^6 to 5.6×10^4 ergs · cm^{-2} · s^{-1} by means of steel-net screens or neutral density filters (●) (From Casadio et al., 1978, reproduced with the kind permission of Dr. Casadio, FEBS and Elsevier)

Table 1

Effect of inhibition of succinate oxidation by malonate on the rate of ATP synthesis and the size of the protonmotive force generated by submitochondrial particles

Expt.	Malonate	Respiration rate ng.atom of $O_2 \cdot min^{-1} \cdot mg^{-1}$	Inhibition %	ATP-synthesis rate $nmol \cdot min^{-1} \cdot mg^{-1}$	Inhibition %	$\Delta\tilde{\mu}_H$ mV
1	−	166	−	83	−	185
	+	93	44	41	50	185
2	−	161	−	78	−	175
	+	87	46	36	54	180
3	−	225	−	51	−	195
	+	115	55	23	57	185
4	−	150	−	93	−	175
	+	60	58	28	70	188

The flow dialysis cell contained in a total volume of 1 ml: 10 mM-HEPES sodium salt, 200 mM sucrose, 2 mM $MgCl_2$, 50 mM KCl, 10 mM KH_2PO_4, 20 mM glucose, 0.17 mM ADP, submitochondrial particles (approx, 9 mg of protein) and 25 U of salt-free hexokinase. The initial concentration of sodium succinate was 20 mM and where added the sodium malonate concentration was 0.95 mM. For determination of $\Delta\tilde{\mu}_H$, potassium thiol(^{14}C) cyanate $\mu(6\mu$ M) or (^{14}C) methylamine (15 μM) were additionally added, and for measurement of the rate of ATP synthesis 2 μCi of $^{32}P_i$ was present. The rate of ATP synthesis was linear over the 8 min period tested in Expts. 3 and 4 whereas in Expts. 1 and 2 a slight initial lag was observed, and thus the averaged rate over 8 min is given. The temperature was 25 °C and the pH was 7.5. The submitochondrial particles were preincubated with 5 mM sodium succinate for at least 10 min at 25 °C to activate succinate dehydrogenase. (From Sorgato et al., 1980, reproduced with kind permission of Dr. Sorgato and The Biochemical Society)

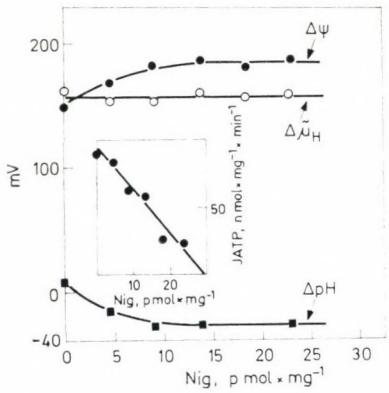

Fig. 6. The effect of nigericin (nig.) on $\Delta\tilde{\mu}_H$ and (insert) on the rate of ATP synthesis. Experiment was carried out as in Table 2, except that the ADP concentration was 0.5 mM and (insert) 1.5 mM (From Zoratti et al., 1981, reproduced with kind permission of Dr. Zoratti, FEBS and Elsevier)

either side of the membrane in the presence of valinomycin. Therefore, such an error in $\Delta\Psi$ measurement seems unlikely.

ad. ii: Casadio et al. (1978) (Fig. 5) showed that the relationship between $\Delta\tilde{\mu}_H$ and phosphorylation rate in chromatophores depended strongly on whether $\Delta\tilde{\mu}_H$ was varied by varying light intensity or by varying proton permeability, leaving L_p, and ΔG_p constant. Sorgato et al. (1980, Table 1) showed that 50% malonate-inhibition of 'state 3' oxidation of succinate by submitochondrial particles inhibited phosphorylation rate by some 50%, without a significant effect on $\Delta\tilde{\mu}_H$. Zoratti et al. (1981) (Table 2, Fig. 6) showed that in rat liver mitochondria phosphorylation may be inhibited by nigericin (an H^+/K^+ exchanger) without a concomitant decrease in $\Delta\tilde{\mu}_H$ and that on the other hand $\Delta\tilde{\mu}_H$ may be decreased without a significant (or with a wrongly directed) effect on phosphorylation.

Table 2

Relationship between phosphorylation rate and $\Delta\tilde{\mu}_H$ at varying external K^+-concentrations in valinomycin treated mitochondria

K^+ mM	J_P nmol·mg^{-1}·min^{-1}	$\Delta\tilde{\mu}_H$ mV
0.3	98	197
0.5	91	193
1.0	114	182
2.0	111	171
3.0	110	162
5.0	102	149

Medium composition: 0.18 M sucrose, 10 mM choline Chloride, 5 mM Tris-MOPS, 3 mM P_i-Tris, 10 mM succinate-Tris, 0.3 mM ADP, 2 μM rotenone, 1 mM EDTA-Tris, 23 pmol/mg valinomycin, KCl as indicated, pH 7.4, 303 K. J_P was determined from the concentrations found in a suspension of rat liver mitochondria (6 mg/ml) quenced with $HClO_4$ 20 s after valinomycin addition. $\Delta\Psi$ and ΔPH were determined from the distribution of (^{14}C)--TPMP$^+$ and (^{14}C)-DMO respectively after 20 s incubation. (From Zoratti et al., 1981, reproduced with the kind permission of Dr. Zoratti, FEBS and Elsevier)

Table 3

The relationship between $\Delta\tilde{\mu}_H$ and the energy charge (E. C.) in intact Halobacterium halobium cells under different energization conditions

(From Helgerson et al., unpublished)

External salt	pH	Light	Addition	$\Delta\Psi$ mV	ΔP_H mV	$\Delta\tilde{\mu}_H$ mV	E.C.
3 M KCl +1.25 M NaCl	6.0	−	TPT	<15	<15	<15	0.39
		+	TPT	85	<15	85	0.93
		+	TPT + CCCP	<15	<15	<15	0.96
		−	TPT + CCCP	<15	<15	<15	0.50

Comments. *(J. Korenbrot)*: In concluding from $J_p = J_p$ (L_p, ΔG_p, $\Delta\tilde{\mu}_H$) that J_p is not expected to vary when an agent is introduced that changes ΔG_p, nor $\Delta\tilde{\mu}_H$, it is assumed that this agent does not directly affect the activity of the $\vec{H}^+$-ATPase (L_p)!

(H. V. Westerhoff): That is right; the consequences *(i)* and *(ii)* depend on the absence of such 'allosteric' (i.e. not energy transducing) interactions. Hence the above experiments could all be explained by postulating the presence of such allosteric interactions (cf. the dotted lines in Fig. 3) superimposed on the chemiosmotic coupling scheme of Fig. 2A. For allosteric influence of redox state and uncouplers (Hanstein, 1976), on $\vec{H}^+$-ATPase activity (Mill, Mitchell, 1982) there is some experimental evidence. Allosteric effects of nigericin, malonate and ADP (in as far as relevant for these experiments) are ill-documented.

ad. iii: Decreasing $\Delta\tilde{\mu}_H$ with protonophore or respiratory inhibitor (Westerhoff et al., 1981b) increased the $\Delta G_p/\Delta\tilde{\mu}_H$ ratio in rat liver mitochondria (Fig. 7)- The analogous result was found for reversed oxidative phosphorylation in sub. mitochondrial particles (Fig. 8).

Comments. *(M. Wikström)*: If you add a protonophore to rat liver mitochondria in state 4, then it is quite likely that $\Delta\tilde{\mu}_H$ decreases *much* more rapidly than ΔG_p just because of the adenylate kinase reaction: $2ADP \rightleftarrows ATP +$ + AMP which buffers the ATP/ADP ratio.

(H. V. Westerhoff): Experiments starting with AMP gave the same results (cf. Westerhoff et al., 1981b).

ad. iv: Figure 9a shows what effect addition of some oligomycin (inhibitor of $\vec{H}^+$-ATPase) should have on the titration of reversed oxidative phosphorylation if with an inhibitor of the electron chain, the high energy intermediate would be shared by a number of $\vec{H}^+$-ATPases and respiratory chains ('pool function'): After a 50% inhibition of the $\vec{H}^+$-ATPase the respiratory chain would be expected

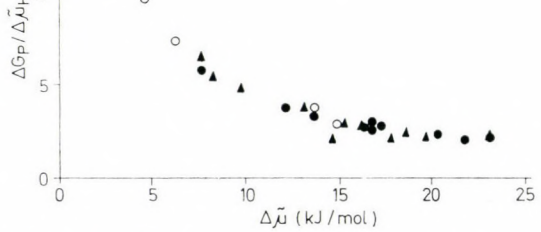

Fig. 7. Dependence of 'state 4' $\Delta G_p/\Delta\tilde{\mu}_H$ on $\Delta\tilde{\mu}_H$ with succinate as substrate $\Delta\tilde{\mu}_H$ is varied either with 0—33 mM malonate (●, ○) or with protonophore (FCCP 0—002 nmol/mg of procein) (▲). ● and ▲ are from one experiment. ○ is from an essentially identical experiment using ^{86}Rb and $|^{14}C|$5.5-dimethyloxazolidine-2,4-dione distribution to measure $\Delta\mu_H$. Rat liver mitothondria. 1 kJ/mol corresponds to 10.4 mV (Cf. Westerhoff et al., 1981b, with kind permission of the Biochemical Society)

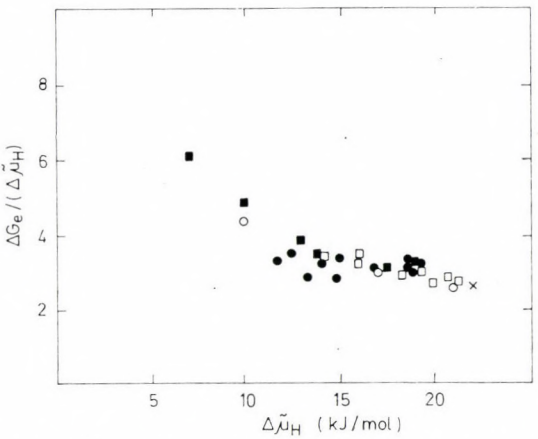

Fig. 8. The dependence of the 'state -4' ratio of the redox potential difference between NADH/ /NAD and the succinate/ fumarate couple to $\Delta\tilde{\mu}_H$ on $\Delta\tilde{\mu}_H$ in ATP energized reduction of NAD by succinate in submitochondrial particles. $\Delta\tilde{\mu}_H$ was varied by adding varying amounts of protonophore (FCCP, ■), oligomycin (○), or varying the amounts of adenine nucleotides (□), or (●) creatine kinase. (×): with maximum creatine kinase without further addition. Experimental conditions as in De Jonge and Westerhoff (1982) (Cf. Westerhoff et al., 1983) ΔG_e is per 2 electrons

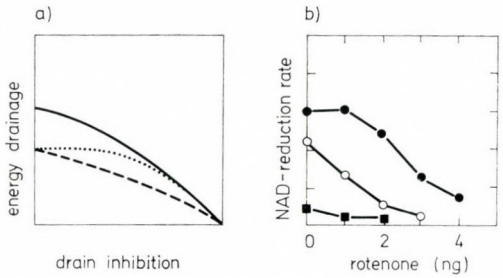

Fig. 9. The effect of partial inhibition of 'site 1 reversal' (i.e. ATP energized reduction of NAD by succinate) in submitochondrial particles with oligomycin on its titration with rotenone (inhibitor of the respiratory chain). (a) Without oligomycin (—) and with as expected when the high energy intermediate is a pool delocalized (...), or localized (---) (b) as found experimentally (cf. Baum et al., 1971; Westerhoff et al., 1983a, b). A quartz cuvette containing 2.0 ml of 0.15 M sucrose, 10 mM HEPES, 50 mM KCl, 5 mM K_2HPO_4, 1.0 mM EGTA, 10 mM $MgCl_2$, 1.0 mM Na_2H_2ATP, 165 μg protein of submitochondrial particles (De Jonge, Westerhoff, 1982) plus no (●), 20 (○), or 40 (■) ng oligomycin was preincubated for 3 h on ice, pH 7.4. Then the cuvette was preincubated for 2.0 min in the 298 K-thermostated cuvette holder of the Amino-Chance dual wavelength spectrophotometer (A_{575}-A_{550}), after which 5 mM succinate, 1.0 mM cyanide, 1.25 mM PEP, 2 μl (20 μg, Boehringer) pyruvate kinase and 0.5 mM NAD were added. Half a minute later a sequential titration with the indicated amounts of rotenone was started. The rate shown is that of NADH formation in relative units (linear scale). The highest rate was about 0.09 μmol NADH · min^{-1} · mg $prot^{-1}$ (From Westerhoff et al., unpublished)

to have overcapacity, so that the first amount of added rotenone would be expected to be without effect (Baum et al., 1971; Hitchens, Kell, 1982a; Westerhoff et al., 1983). The experimental results (Fig. 9b) suggests that inhibition of the $\vec{H}^+$-ATPase makes the respiratory chain more, rather than less rate limiting. Hitchens and Kell (1982a) found similar results in phosphorylation in chromatophores (Fig. 10).

Comments. *(M. Wikström)*: Does not the proposed interpretation of these dual inhibition titration studies depend on the assumption that the extent of binding of the inhibitor is not affected by the energy state ($\Delta\tilde{\mu}_H$)?

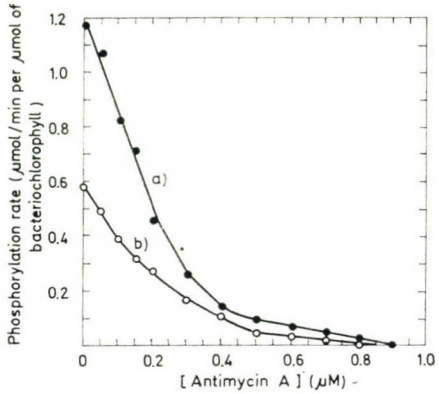

Fig. 10. The effect of DCCD and antimycin A on phosphorylation by *Rps. capsulata* chromatophores without (a) and with (b) 50 mM DCCD. (From Hitchens, Kell, 1982a, reproduced with kind permission of Dr. Kell and the Biochemical Society)

(H. V. Westerhoff): Yes, it does, and indeed for one of the inhibitors used (antimycin A) such energy dependence of the binding has been suggested in the literature. The experiment of Fig. 9b has been carried out with prolonged preincubation with oligomycin with *essentially* the same result as with shorter preincubations (cf. Westerhoff et al., 1982, 1983). The remaining problem is the rotenone. The constancy of its binding should be checked.

(M. K. F. Wikström): The interpretation also strongly depends on the assumption that the inhibitors used are strictly specific. For the experiments of Hitchens and Kell (1982a) this assumption has recently been shown to be incorrect: DCCD also affects the bc_1 complex (Price, Brand, 1982).

ad. v: Kraayenhof and Slater (1974) showed that atebrin coupled to sepharose and hence unable to penetrate the membrane, still increased photophosphorylation, unless it was allowed to sediment (cf. Fig. 11).

Comments. *(J. Korenbrot)*: Could not one explain these results by assuming that the atebrin-sepharose inhibited phosphorylation?

(H. V. Westerhoff): It was shown that the atebrin sepharose acted like an uncoupler in the sense that 'state 2' electron transport was stimulated and photophosphorylation was inhibited (Fig. 11). It has however not been checked whether it would also inhibit ATP hydrolysis (Kraayenhof, personal communication).

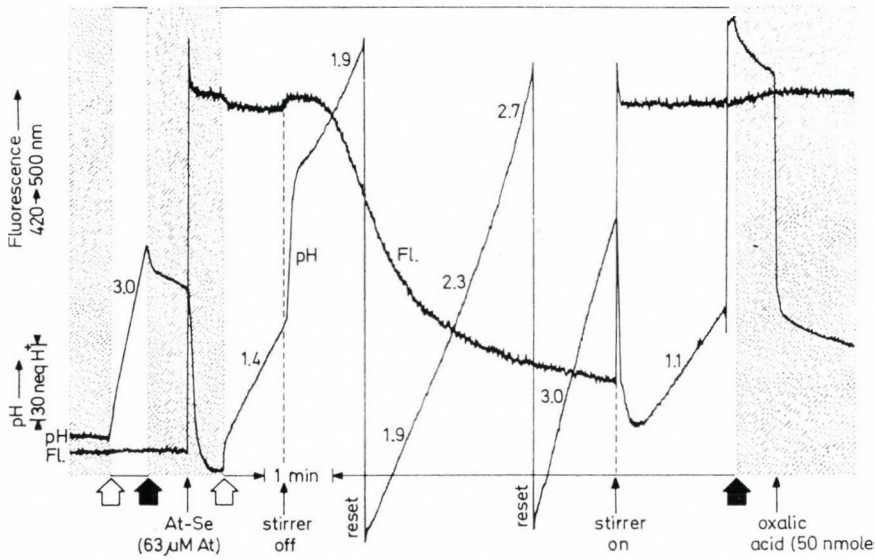

Fig. 11. Reversible inhibition of photophosphorylation in chloroplasts by atebrin-Sepharose conjugate. Medium 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 2 mM P_i, 1 mM Tricine, 10 μM pyocyanine and 40 μg/ml Chlorophyll, pH 8.0, 293 K. The rates of ATP synthesis (measured as change in pH) are expressed in μmol · min^{-1} · mg Chlorophyll^{-1} (From Kraayenhof and Slater, 1974, reproduced with kind permission of Dr. Kraayenhof, FEBS and Elsevier)

ad. vi: Fig. 12 shows that upon partial inhibition of the energy consuming proton pump in site 1 reversal in submitochondrial particles less, rather than more protonophore is needed to eliminate the energy consuming reaction. Hitchens and

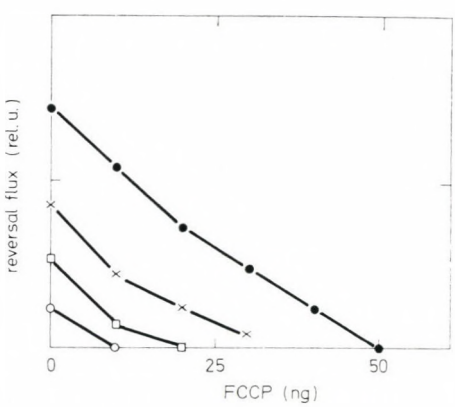

Fig. 12. The effect of rotenone addition on the amount of protonophorous uncoupler needed to stop ATP-energized reversed electron transfer at 'site 1' of oxidative phosphorylation in submitochondrial particles. Conditions as in Fig. 9B, titration with FCCP being preceded by the addition of 0 (●), 2 (×), 4 (□), or 6 (○) ng of rotenone (from Westerhoff et al. unpublished)

Kell (1982b) obtained the analogous result in photophosphorylation in bacterial hcromatophores.

Comments. *(M. Wikström)*: Essentially the same objection as against the type-iv experiment can be made here. If one would suppose that rotenone would become more and more effective, the more FCCP is added, then one would predict this result.

(H. V. Westerhoff): Yes, if one would suppose that at the point of zero reversal rate, all inhibition is due to rotenone binding and none to $\Delta\tilde{\mu}_H$ definiency. However to explain the results in Fig. 9b by an effect of the energy state on the binding affinity, the affinity would have to *increase* with energisation. Here it would have to decrease.

II. Some additional recent data

A. *Halobacterium halobium photophosphorylation: experimental conditions for demonstrating the ΔG_p and $\Delta\tilde{\mu}_H$ can be uncoupled* (by S. L. Helgerson)

In the dark under nitrogen *H. halobium* intact cells containing the light-driven proton pump bacteriorhodopsin (bR) maintain a preexisting membrane potential ($\Delta\Psi$) and pH gradient (ΔpH). The total protonmotive force is -150 mV. In the light both of these parameters increase. We can measure $\Delta\Psi$ and ΔpH using flow dialysis of [^{3}H]-TPMP$^+$ and [^{14}C]-benzoic acid. With the ionophores triphenyltin (TPT) and CCCP both ΔpH and $\Delta\Psi$ can be collapsed. TPT acts by exchanging Cl^- and OH^- across the membrane so that ΔpH collapses both in the dark and light, while $\Delta\Psi$ is unaffected (in the dark but is enhanced somewhat in the light). After TPT, the protonophore CCCP can be added to collapse $\Delta\Psi$. Using flow dialysis (Lanyi et al., 1979) and/or centrifugation through silicon oil (Michel, Oesterhelt, 1976), we measure that in the presence of TPT and CCCP $\Delta\Psi$ and ΔpH must be <15 mV and are probably zero. This is true for both dark and illuminated cells. *H. halobium* cells normally have internal salt concentration of 3–3.5 M KCl and 1 M NaCl while the external medium is 4 M NaCl and 30 mM KCl. To lower any contribution from these ion gradients, the cells are resuspended in an external solution containing 3 M KCl and 1.25 M NaCl.

Both the rate and extent of ATP synthesis are controlled by the light intensity. In the experiments described below, the steady state levels of ATP, ADP, AMP, and P_i were measured and the phosphorylation potential (ΔG_p) and energy charge of the system calculated. The energy charge parameters E.C. (defined as (2[ATP] + [ADP])/([ATP] + [ADP] + [AMP]) provides important information: (a) the percentage that each nucleotide contributes to the total nucleotide pool is known, (b) the total size of the nucleotide pool can be shown to be constant under all experimental conditions, and (c) the presence and apparent equilibrium constant of the endogenous adenylate kinase reaction can be measured. We have shown that all photophosphorylation is inhibited in cells pretreated at 0 °C with a low concentration (25 μM) of DCCD. Also, strains which lack bacteriorhodopsin

show no photophosphorylation activity under the conditions of the experiments, even though respiration (glycerol + O_2) and substrate level phosphorylation activities are unaffected.

The experiment then is to measure the steady state levels of ATP, ΔG_p, E.C., $\Delta\Psi$ and ΔpH in cells under N_2 with and without illumination. From this we can test whether or not ΔG_p and $\Delta\tilde{\mu}_H$ are coupled. Both TPT and CCCP effect the rate of ATP synthesis. This would be expected if they lower $\Delta\tilde{\mu}_H$ or if they act to increase the membrane permeability to protons. However, in the steady state ΔG_p would be affected only if $\Delta\tilde{\mu}_H$ is coupled to ΔG_p. Some of the results are given in Table 3. The energy charge values reflect both the level of ATP and ΔG_p, i.e. low E.C. indicates low ATP and ΔG_p while high E.C. is high ATP and ΔG_p. Similar experiments were done at other pH values and external salt combinations. In all cases the main result could be demonstrated.

The E.C. (or ΔG_p) values in the dark are insensitive to $\Delta\tilde{\mu}_H$ because it is the adenylate kinase reaction which maintains this level of ΔG_p. That is to say the $\vec{H}^+$-ATPase does not synthesize ATP in the dark in response to $\Delta\tilde{\mu}_H$. The insensitivity of ΔG_p to $\Delta\tilde{\mu}_H$ in the light is a matter of speculation. One possibility would be that bacteriorhodopsin pumps enough protons to drive ATP synthesis directly. This might occur by building up a large concentration difference of protons locally across the membrane surface. Thus, the driving force might be a proton current rather than a proton electrochemical gradient. It is clear that at least under some experimental conditions ΔG_p and $\Delta\tilde{\mu}_H$ are not directly coupled in *H. halobium*. A similar conclusion was reached by Michel and Oesterhelt (1980).

B. *The effect of low concentrations of uncouplers on proton release during flash-induced photosynthetic electron transport* (by S. Theg)

Even though the half time for proton equilibrium across the thylakoid membrane (bulk to bulk) is approximately 10 s (Auslaender, Junge, 1975), thylakoids are thought to maintain a proton gradient in the dark for as long as 10 min (Baker et et al., 1981; Theg, Homann, 1982; Theg et al., 1982). This gradient ($\Delta\tilde{\mu}_H$) can be collapsed by adding uncouplers to suspensions of chloroplasts in the dark (Baker et al., 1981; Theg, Homann, 1982; Theg et al., 1982). We wanted to know how the removal of the $\Delta\tilde{\mu}_H$ would affect electron transport-dependent proton deposition into the thylakoid lumen. Accordingly, we investigated the effects of low concentrations of uncouplers on the flash-induced absorption changes of neutral red in chloroplast suspension. As shown previously (Junge et al., 1979; Hong, Junge, 1983), under conditions of selective buffering of the external medium neutral red monitors the proton concentration at the inner membrane surface, which is in rapid equilibrium with those in the bulk aqueous (and osmolar) lumen.

We found that: 1. Gramicidin, FCCP, A23187 and nigericin rendered the protons derived from water oxidation by photosystem II (rapid phase of acidification of the lumen, cf. Auslaender, Junge, 1975) undetectable by neutral red, while causing no, or very little, change in the observable proton deposition during plastoquinol oxidation (slow phase of acidification). Results for gramicidin are

shown in Fig. 13. 2. Concentrations of the non-protonophoric ionophores valinomycin and ETH 1001 which produced roughly the same increase in electrical conductivity of the membrane as gramicidin did not affect the rapid phase of proton deposition. 3. With gramicidin, the effect was pH dependent and satarated at an uncoupler concentration of 1 nM. 4. The neutral red signal arising from protons released during PMS-mediated cyclic electron flow around photosystem I (+ DCMU) was unaffected by gramicidin. 4. The extent of alkalinization of the external medium during flash-induced electron transport from water to benzylviologen was unaffected by gramicidin. This last observation shows that the undetected water-derived proton had not been released to the external medium. Observations 4 and 5 respectively indicate that gramicidin does not alter the buffering capacities of either the lumen or the external phase.

Building on the hypothesis of Dilley and his co-workers (Baker et al., 1981), it is postulated: 1. Protons derived from water oxidation are initially deposited into special domains located within the thylakoid membrane. 2. In the dark, the domains are isolated from both bulk phases by nearly proton-impermeable barriers. Thus, $\Delta\tilde{\mu}_H$(bulk) does not necessarily equal $\Delta\tilde{\mu}_H$(domain) unless uncouplers are added. 3. Protonatable buffering groups reside in the domains. 4. Only those protons which are not bound by buffering groups after a flash are rapidly released from the domains to the lumen. It is proposed that, in the absence of gramicidin, the special domains are saturated with protons which originated in earlier exposures to light and which could not equilibrate with the bulk aqueous phases across the domains' high proton permeability barrier. Protons subsequently entering the domains after a flash would therefore encounter relatively few unprotonated buf-

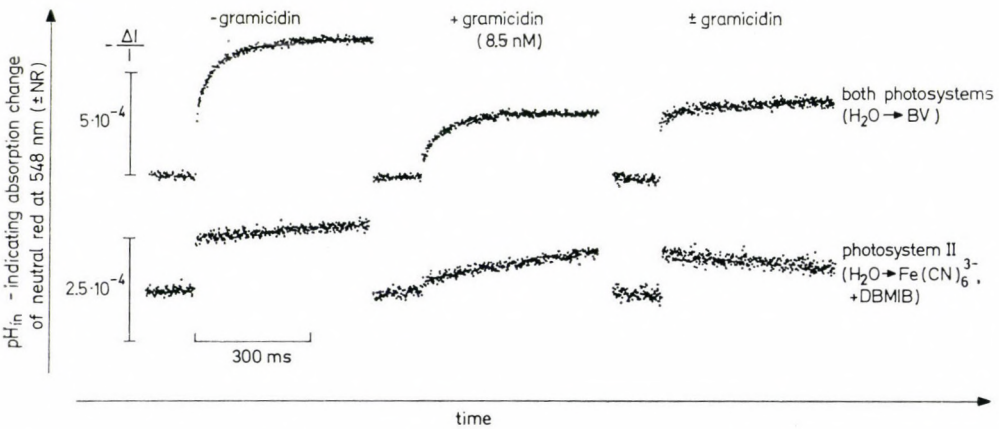

Fig. 13. Effect of gramicidin on flash-induced internal proton release form both photosystems (above) and from photosystem II alone (below). Left-hand and middle traces were obtained by signal averaging (20–40 flashes separated by 6 s). Right-hand traces were obtained by subtracting the *plus* gramicidin curves from the appropriate controls. The rapid phase of the neutral red signal corresponds to water-derived protons, the slow phase to protons from plastoquinol

fering groups and would quickly pass into the lumen and be detected as the rapid phase of the neutral red signal. In the presence of gramicidin (or another protonophore) and at alkaline pH, the domains empty out into the bulk phases during the dark periods between flashes, provided that these dark periods are sufficiently long. Protons pumped into the domains upon flash excitation would then be bound by the unprotonated buffering groups and not enter the lumen: no rapid neutral red response would be seen.

Such a model predicts that the original amplitude of the neutral red signal should be recoverable if protons are deposited within the domains faster than they are re-equilibrated by gramicidin. This was observed when a series of flashes spaced 150 ms apart were delivered to the chloroplasts. As seen in Fig. 14, no rapid proton deposition (attributable to water-derived protons) was observable in the first few flashes, but developed gradually in subsequent few flashes. The inset

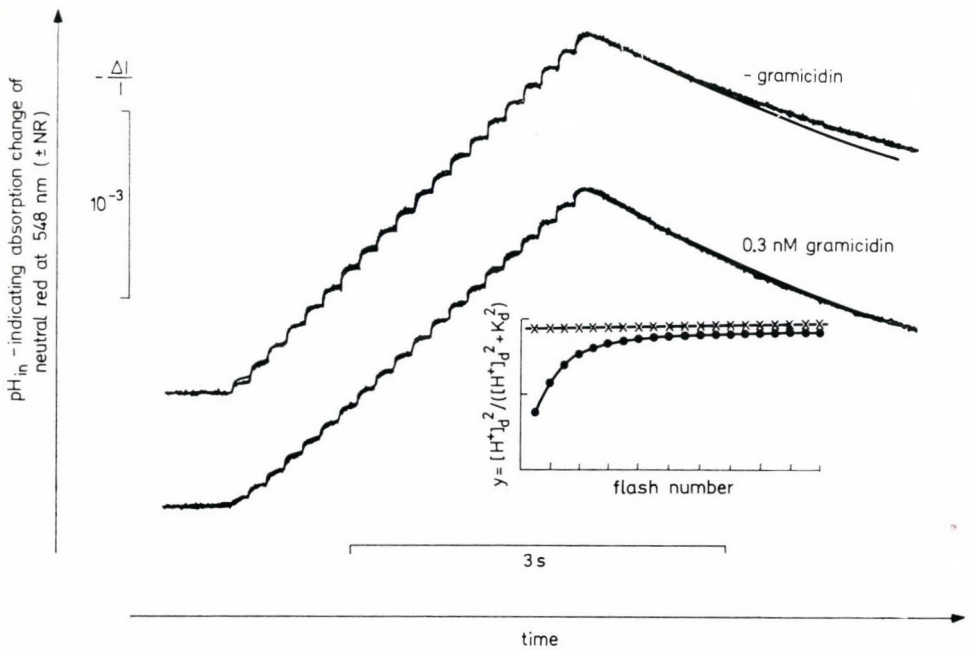

Fig. 14. Absorption changes of neutral red during H_2O to benzylviologen electron transport driven by a sequence of 20 closely spaced flashes. (a) no gramicidin; (b) plus 0.3 nM gramicidin. Chloroplasts flashed at a frequency of 6.67 Hz. Lines with noise are the actual data from the signal averager (5 sets of flashes separated by 30 s). The smooth lines were calculated from a model in which water-derived protons were assumed to enter a highly buffering domain, from which a fraction, γ, passed to the lumen. γ is unity when the domain's buffering capacity is saturated. Inset: Calculated relative contribution of the water-derived proton to the fast phase of the neutral red signal. ×, no gramicidin; •, + gramicidin. The divisions on the abscissa mark every second flash; the ordinate is scaled from 0 (no rapid phase) to 1 (rapid phase fully developed). $[H^+]_d$ corresponds to the concentration of free protons in the domains, and K_d to the apparent asscciation constant of the domains' buffering groups. (For details of the calculation see Theg, Junge 1983)

shows that the amplitude of the rapid phase recovered 90% of the control value by the seventh flash. This gramicidin-induced proton deficit corresponded to approximately 12 nmol H^+/μmol Chl, a number comparable to estimates of the size of the dark-held proton pool derived by other methods, i.e., 10–100 nmol H^+/μmol Chl (Baker et al., 1981; Theg et al., 1982).

It is noteworthy that not all chloroplast preparations gave the above described results. For instance, chloroplasts which had been stored for two years in liquid N_2 with DMSO as cryoprotective, and which were still competent for O_2 evolution and photophosphorylation, did not show differential sensitivity of water- and plastoquinol-derived protons to gramicidin.

The experiments described above suggest that special proton sequestering domains may exist within the thylakoid membrane. Even though their relation to energy coupling has not been demonstrated, their existence has been anticipated by hypotheses of localized chemiosmosis, and thus may have some bearing on the mechanism of energy transduction in chloroplasts.

A complete description of these experiments has been accepted for publication (Theg, Junge, 1983).

Two criticisms of this work were offered at the time of its presentation in Szeged. First, we cannot be sure that neutral red is a clean indicator of pH changes in the thylakoid lumen. This point has been specifically investigated by Junge and his co-workers (Junge et al., 1979; Hong, Junge, 1983), including myself (unpublished). The experiments suggest that neutral red does not respond to changes in the transmembrane potential or undergo redox reactions in thylakoid suspensions, but rather responds to pH changes in an osmotically active space which is accessible to certain hydrophobic buffers. Presumably this space is the thylakoid lumen.

Second, it was suggested that ions other than protons may be released during flash-induced electron transport in the presence of gramicidin, thus preserving the proton's usual contribution to the delocalized $\Delta\tilde{\mu}_H$ as an electrical potential. This seems unlikely from measurements of the field-indicating electrochromic absorptions changes of carotenoids made in the presence of gramicidin (experiments not shown). These measurements revealed that the magnitude of the potential resulting from the flash-induced primary charge separation was not altered by gramicidin: no additional potential was detected. The possibility that such an additional potential might have been offset by the loss of an electrical contribution of proton deposition into the lumen could be excluded since the latter event is not in itself electrogenic (Auslaender, Junge, 1975; Foerster et al., 1981).

C. *Flash-induced absorption change at 515 nm in chloroplasts as a monitor for localized chemiosmosis* (by O. van Kooten)

The flash-induced absorption change at 515 nm in plant leaves or chloroplasts shows multiphasic kinetics. In a recent article (Vredenberg, 1981) the different interpretations of this phenomenon have been reviewed. None of these interpretations is capable to explain the large variety of experimental results obtained in the past 15 years. I have developed a model (see the last part of this paper)

which can explain the experimental data. The model is based on our deconvolution of absorption change kinetics of P515 (Schapendonk, 1980). We deconvolute the P515 response into two major components 'Reaction I' and 'Reaction II'.

Reaction I has a fast rise time <0.1 μs and a single exponential decay with a $\tau_{1/2} = 40$ ms. We have been able to correlate Reaction I with the transmembrane potential and its dissipation through ion diffusion across the membrane. Reaction II consists mainly of two exponentials. One rising with a $\tau_{1/2} = 60$ ms and one decaying with a $\tau_{1/2} = 400-600$ ms. The decay kinetics of Reaction II are too slow to be explained by ion diffusion across the membrane. With DCCD experiments in spinach leaves, we have been able to correlate Reaction II to ATP synthesis. Other laboratories have also correlated Reaction II with the activity of the ATP synthesizing enzyme (Schuurmans et al., 1981, Schreiber, Reinits, 1982). The data can be explained by assuming that Reaction II reflects an intramembraneous electrical event caused by localizing charges (e.g. protons) which cannot easily equilibrate with the lumen.

III. Criticism on I and II

(contributed by Wikström, Westerhoff and all present)

The following Table was constructed:

Number*	Type of experiment Description	Probl ems with the interpretations of the experiment
i & ii	$J_0 \neq f(L_0, \Delta G_0, \Delta\tilde{\mu}_H)$ $J_p \neq f(L_p, \Delta G_p, \Delta\tilde{\mu}_H)$	Measurement of $\Delta\tilde{\mu}_H$ Allosteric interactions
iii	$\Delta G_p/\Delta\tilde{\mu}_H$ varies with $\Delta\tilde{\mu}_H$ (also Sam Helgerson)	Measurement of $\Delta\tilde{\mu}_H$ (but in cases $\Delta\Psi \equiv 0$)
iv	No pool behaviour	Energy dependence of the binding of the inhibitors
v	Non-shuttling uncoupling substances	Is it really bound to the Sepharose? Inhibition of $\tilde{H}^+$ ATPase
vi	Less uncoupler needed at higher $\Delta\tilde{\mu}_H$	Energy dependence of the binding of the inhibitor
vii	Protons of photosystems I and II are differently buffered away (Steven Theg)	Neutral red does also read local charges (Bogomolni, Stoeckenius)
viii	One flash saturable proton capacity (Olaf van Kooten)	Kinetic fit risky Electric field decay due to movement of more than one ion species The generated $\Delta\Psi$ itself may slow down kinetics

* correspond to the same paragraphs in part I.

IV. Alternatives to delocalized chemiosmotic coupling

A. *General properties* (by H. V. Westerhoff)

Figure 15 illustrates which elements an alternative chemiosmotic hypothesis for energy coupling would have to contain (cf. Van Dam et al., 1978; Westerhoff et al., 1983; for references to similar, earlier proposals see Westerhoff et al., 1984b):

(i) The high energy intermediate does not behave like a pool and therefore coupling is catalyzed by a large number of small units, each of which contains a very small number (e.g. 1) of redox energy coupling enzymes and a similar number of F_0F_1-ATPases.

(ii) Redox chains and F_0F_1-ATPases pump protons, but into a small space that belongs to their special unit (see above). There is some barrier that keeps the protons (or other ions) from rapidly equilibrating with the bulk phase.

(iii) The energy coupling membrane is essentially not spherically symmetric: there is a proton leakage pathway from bulk phase to bulk phase at certain positions in this membrane, distinct from the special energy coupling units. Kell et al. (1981, cf. Fig. 16) suggested that the above proposed units would be functional rather than topological; redox chains and $\vec{H}^+$-ATPase would be interconnected

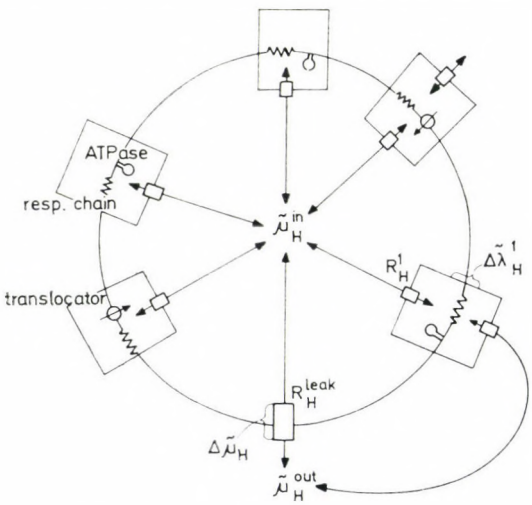

Fig. 15. Minimum hypothesis for chemiosmotic energy coupling: MOSAIC CHEMIOSMOSIS. Every Free Energy source (respiratory chain proton pump) is functionally connected to a Free Energy sink ($\vec{H}^+$-ATPase, $\vec{H}^+$-translocator, etc.) thus forming an energy-coupling unit. Within each unit i a local $\Delta\mu_H$ ($\Delta\tilde{\mu}_H^i$) is (but see Westerhoff et al., 1983) the high energy intermediate. The $\Delta\tilde{\lambda}_H$'s do not readily equilibrate with the bulk phase to bulk phase electrochemical potential difference for protons ($\Delta\mu_H = \mu_H^{in} - \tilde{\mu}_H^{out}$), owing to the presence of a resistance R_H^i for (proton) current between the energy coupling unit i and the bulk phase, and to the presence of a proton leakage pathway for protons from bulk phase to bulk phase (R_H^{leak}) (Cf. Westerhoff et al., 1982, 1983, 1984)

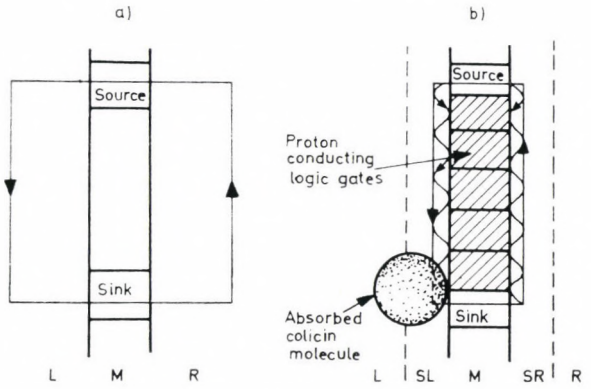

Fig. 16. Diagrammatic representation of two (purely 2 dimensional) models for the proton current pathway of membrane energy coupling. In the more classical chemiosmotic respresentation (a) the membrane is viewed as a passive diffusion barrier (M phase) separating two aqueous phases (L and R) wherein the proton current is carried. In an alternative view (b), however, the proton current is carried within the membrane/solution interphases (SL an SR), aided by conformationally switchable protonic 'logic gates', constituting a protoneural network. A single adsorbed colicin molecule, by binding to an element of such a network, could cause a cooperative conformational transition in this network, thereby inhibiting energy coupling. Arrows represent the flow of protonic current (From Kell et al., 1981, reproduced with the kind permission of Dr. Kell, FEBS and Elsevier)

by specific proton wires. Of the other, similar, coupling hypotheses (reviewed in Westerhoff et al. 1984b), the ones of Willams (1962), Ernster (see Boyer et al., 1977) and Dilley et al. (1982) should be mentioned in this connection. Olaf van Kooten will now suggest another tie between redox chain H^+-ATPase.

B. *A model explaining the electrochromic absorption change at 515 nm in terms of Reaction I and Reaction II* (by O. van Kooten)

As I have debated above, Reaction I reflects the onset and decay of the transmembrane potential. It is caused by those charges which can equilibrate with the water phases on both sides of the thylakoid membrane directly after the charge separation. Since the conductivity of the lumen is very high this will immediately be sensed by the carotenoid as a field over a flat capacitor C_m which is dissipated through a resistor R_m in parallel (see Fig. 17a). On the other hand those charges that cannot equilibrate with the lumen, because there is a hydrophobic barrier restricting their movement into the water phase, will give rise to completely different field kinetics near the carotenoid. Those charges can be assumed to reside on a capacitor C_p (cf. Fig. 17b). They will give rise to a strongly localized electric field. They are able to equilibrate with the stroma through a large resistor R_p Depending on its distance the carotenoid will not be able to sense this localized

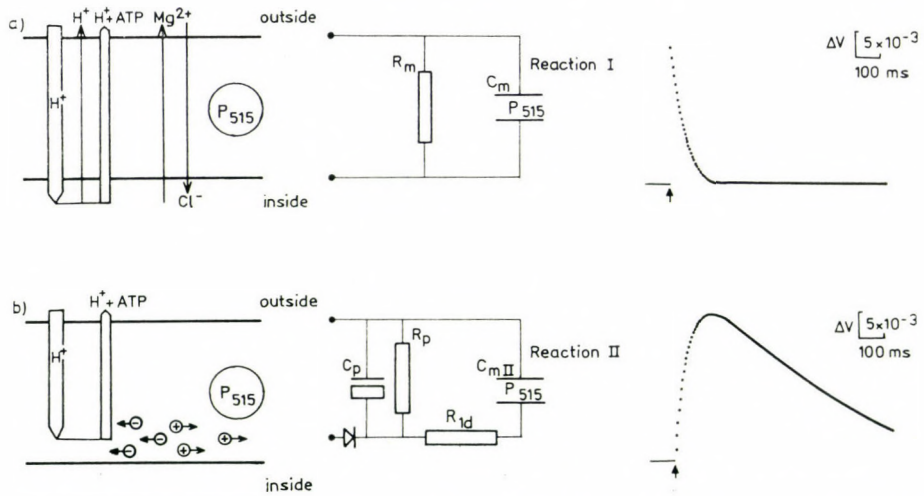

Fig. 17. a: Schematic view of transverse charge transport in the thylakoid membrane with its electrical network equivalent. The potential over the capacitor C_m is correlated with the field sensed by P515 (Proposed by Van Kooten) b: Schematic view of transverse and lateral charge transport in the case that the transported protons are unable to equilibrate with the water phase inside the thylakoid (Proposed by Van Kooten)

field directly. The membrane is filled with proteins which have charged and polar residues even within the hydrophobic space. These residues will cause lateral electrophoretic movement of the charged proteins and thereby delocalize the field. This lateral vectorial movement will be restricted by the random diffusional motion and by the membrane viscosity. The electrophoretic motion of the charged molecules can be considered as moving through a resistor R_{Ld} (lateral diffusion resistor). The part of the membrane that separates these moving charges from the bulk aqueous phase acts as an electric capacitor C_{mII}. This electric circuit, when charged with a short charge pulse Q, will give rise to an electric field in C_{mII} which is kinetically identical with Reaction II (see Fig. 17b).

V. Conclusions

(by Zs. Dancsházy and H. V. Westerhoff)

Although some problems (listed in section III) remain to be solved, a number of experimental results cannot be understood in terms of a chemiosmotic coupling theory in which the high-energy protons are delocalized over a bulk aqueous phase (see also Westerhoff et al., 1983, 1984). The possibility that coupling occurs through the operation of a large number of miniature chemiosmotic coupling devices (see also Mitchell, 1977, Westerhoff et al., 1983), should be further investigated. It may then also be of interest to estimate the relevance of elastic, Coulombic and

polarization energy that may be linked to membrane energization (Williams, 1979). Furthermore, the use of 'local' probes with well-defined positions (bacteriorhodopsin?) to estimate local proton potentials, may be informative (see also Schuurmans et al., 1981 Dancsházy et al., 1983; Helgerson et al., 1982).

VI. The proton cycle and the local field

(by V. P. Skulachev)

The essence of the chemiosmotic hypothesis presented by Mitchell in 1961 (Mitchell, 1961) can be formulated in the following way:

In oxidative and photosynthetic phosphorylations, the energy sources are used by redox enzymes to transport H^+ ions across the hydrophobic barrier of a membrane. Back flow of H^+ ions catalyzed by H^+-ATP-synthetase is coupled to formation of ATP from ADP and P_i.

Bioenergetic studies of the past 20 years have directly confirmed so many qualitative predictions of this concept that now to disprove it one needs facts which *qualitatively* contradict the main Mitchell's postulate.

It is hardly surprising that working with intact cells or organelles one may obtain certain data quantitatively contradicting the simplest concrete version of $\Delta\tilde{\mu}_H$-generating and $\Delta\tilde{\mu}_H$-consuming enzymes, postulated by Mitchell in 1961 – 1966 (Mitchell, 1961, 1966). Natural energy-transducing membranes are so complicated and one's knowledges about mechanisms of enzymes dealing with $\Delta\tilde{\mu}_H$ are so scant that even now we fail to choose the right scheme among many tentative versions of the proton cycle. In this situation, it seems to be reasonable to refrain from postulating novel mechanisms inconsistent with the above formulated proton cycle principle if there is no direct, conclusive, reproducible and qualitative evidence incompatible with this principle.

The above reasoning can be used if we consider "microchemiosmosis", or the "localized $\Delta\tilde{\mu}_H$" concept. This scheme is based on the assumption that an H^+ ion transported across the hydrophobic barrier by a $\Delta\tilde{\mu}_H$-generator (say, a redox-enzyme complex) is consumed by H^+-ATP-synthetase before being diluted by the bulk water phase. In a sophisticated version presented by Kell and Morris (1981), the localized $\Delta\tilde{\mu}_H$-concept suggests that there are special proteins facilitating H^+ movement along the membrane-water interface. Proponents of this point of view proceed, e.g., from evidence that decrease in the $\Delta\tilde{\mu}_H$ level by, say, switching off a portion of $\Delta\tilde{\mu}_H$-generators, results in a situation where the rate of the ATP synthesis becomes proportional to the number of operative generators, rather than to the measured $\Delta\tilde{\mu}_H$ (Kell, Morris 1981; Kell 1979). This might be explained by the assumption that a special H^+-conducting mechanism connecting a $\Delta\tilde{\mu}_H$-generator with the nearest H^+-ATP-synthetase induces a high local ΔpH which supports ATP formation by this H^+-ATP-synthetase, while other molecules of the enzyme remain inactive.

It may be counter-argued that there is no direct evidence of the postulated H^+-conducting mechanism. At the same time, it is quite clear that the H^+ of the

bulk water can reach H^+-ATP-synthetase since artificially-imposed $\Delta\tilde{\mu}_H$ is competent in ATP formation, with no redox-chain enzyme involved (Skulachev, 1981).

In this group, Kara-Ivanov (1983) thoutht of a simpler possibility requiring no lateral H^+ conductance. His idea is that an intricate profile of mitochondrial intermembrane space may be the reason for the formation of a pH-gradient between this space and bulk water space of the incubation medium. This interesting suggestion, however, hardly applies to a suspension of bacterial chromatophores which were also used to study the localized $\Delta\mu H^+$ problem (Venturoli, Melandrini, 1982).

Below I would like to consider another possibility that allows one to do without a special system for lateral H^+ conductance. I shall discuss the role of a local electric field in energy coupling.

An important observation has been made by Teissie et al. who studied ATP synthesis in submitochondrial particles exposed to an electric pulse of different durations (Teissie et al., 1981). It was found that in a 30 kV/cm field, which corresponds to about 200 mV across the membrane of the particles, the critical duration of the pulse was only 8×10^{-6} s. This time is 10^3-fold shorter than the rate of respiratory phosphorylation in submitochondrial particles (Thayer, Hinkle, 1975; Pedersen, 1976) or that of photophosphorylation in chloroplasts (Witt, 1979) and chromatophores (Clayton, Sistrom, 1978).

It is known that there is a threshold in the curve of the H^+-ATP-synthetase rate as a function of the $\Delta\tilde{\mu}_H$ level (Nicholls, 1981).

When the $\Delta\tilde{\mu}_H$ level is lower than the proton-motive force of the $\Delta\tilde{\mu}_H$-generator, it seems reasonable to assume that the local electric field in the vicinity of the generator, which has just transported a proton or an electron, is higher than the delocalized $\Delta\tilde{\mu}_H$.

Such a local field must decrease with time (down to the level of delocalized $\Delta\Psi$) due to spreading along the membrane or, alternatively, to the operation of sinks located near the given $\Delta\tilde{\mu}_H$-generator. As a sink, an adjacent H^+-ATP-synthetase might be used if some local field exists for at least 8×10^{-6} s. This time seems to be realistic when we take into account:

(i) The life-times of separated charges in the $\Delta\tilde{\mu}_H$-generator molecule; and

(ii) The hydrophobic properties of the membrane where the charge separation occurs.

For example, in the reaction centers of chromatophores, charge separation between bacteriochlorophyll (BChl) and Fe-Q results in the former being charged positively and the latter negatively (Clayton, Sistrom, 1978). This process is a partial reaction of $\Delta\tilde{\mu}_H$ generation by the reaction center complex. It takes about 200 ps. The separated charges exist until $BChl^{\cdot +}$ is reduced by cytochrome c, and $Fe\text{-}Q^{\cdot -}$ is oxidized by a secondary quinone ($\tau_{1/2}$ of the overall process is 60–300 μs) (Clayton, Sistrom, 1978). According to our data on the direct measurement of $\Delta\Psi$ formation in chromatophores, BChl-FeQ oxidoreduction is responsible for the major portion of photoelectrogenesis (Drachev et al., 1981). The hydrophobicity of the region where this oxido-reduction takes place (Clayton, Sistrom,

1978) should prevent the fast dissipation of the local field. If there is an H^+-ATP-synthetase near the photosynthetic reaction center complex, a $\Delta\tilde{\mu}_H$-linked stage of ATP-synthesis may be triggered by the local field existing between $BChl^{\cdot+}$ and $Fe\text{-}Q^{\cdot-}$.

Certainly, such a model requires a close proximity of H^+-ATP-synthetase and $\Delta\tilde{\mu}_H$-generator. Apparently, this is usually the case in natural membranes because of a very high concentration of H^+-ATP-synthetase in these membranes. (Remember negative staining of submitochondrial particles: F_1 knobs are so close to each other that it would be impossible to place one more knob in between).

According to Kozlov (Kozlov, 1981; Chernyak, Kozlov, 1979; Kozlov, Skulachev, 1982), $\Delta\tilde{\mu}_H$ performs two functions in the ATP formation:

(i) Energetic, providing a positive overall balance of free energy for the ATP-synthetase reaction; and

(ii) Kinetic, accelerating the slowest stage of this reaction.

The threshold of the ATP-synthesis is due to the second function since below the threshold no formation of ATP, by, e.g., submitochondrial particles, takes place even in the presence of the hexokinase-glucose-6-phosphate dehydrogenase trap when the overall process including ATP synthesis is energy-releasing. The local field may first of all play an important role in the kinetics of H^+-ATP-synthetase. When a delocalized field is below the threshold, the sum of the local $\Delta\Psi$ and delocalized $\Delta\tilde{\mu}_H$ may prove to exceed it, so that the rate of ATP formation will still be high.

It is noteworthy that the above concept does not revise the proton cycle principle since it is assumed that H^+ ions are translocated across the membrane. At the same time, it does not require a mechanism (hardly probable indeed) preventing release of H^+ ion to water after H^+ ion transversed the hydrophobic barrier of the membrane.

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Enzyme-enzyme Interactions within Human Erythrocytes as Suggested from Prelytic Release

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The molecular sieving property of human erythrocyte membrane in slightly hypotonic media has been utilized for studying the intracellular localization of some proteins (Cseke et al., 1978, FEBS Lett. *96* 15—18). It is now shown that four proteins which appear to be uniformly distributed within the cell behave similarly irrespective of the membrane resistance differences caused by changes of temperature and metabolic energy supply.

Five enzymes catalyzing consecutive reactions in the glycolytic pathway between triosephosphate formation and lactate production are released from erythrocytes in quantities deviating from those predicted on the basis of molecular sieving. The data are compatible with the assumption that these enzymes form complexes with each other under in vivo conditions and that complex formation is facilitated if glycolysis is working at high rate (37 °C).

Introduction

Several glycolytic enzymes, glyceraldehyde-3-phosphate dehydrogenase (Kant, Steck, 1973; Solti, Friedrich, 1976), aldolase (Solti, Friedrich, 1976; Strapazon, Steck, 1976), phosphofructokinase (Higashi et al., 1979) and 3-phosphoglycerate kinase (De Barun, Kirtley, 1977) were reported to be complexed with the erythrocyte membrane, more precisely to be bound to hypotonic ghosts. As far as we know, with the exception of 3-phosphoglycerate kinase all of these enzymes were found to bind to the Band 3 protein of ghosts (S rapazon, Steck, 1976; Yu, Steck, 1975; Strapazon, Steck, 1977; Higashi et al., 1979). On the other hand, glyceraldehyde-3-phosphate dehydrogenase binding was claimed to be non-existing under physiological conditions (Maretzky et al., 1974; Fujii, Sato, 1975). Evidence for a surface-near localization of glyceraldehyde-3-phosphate dehydrogenase in intact human erythrocytes was recently reported (Keokitichai,

Abbreviations: CA, carbonic anhydrase; PGK, 3-phosphoglycerate kinase; TPI, triosephosphate isomerase; Hb, hemoglobin; 6-PGDH, 6-phosphogluconate dehydrogenase; LDH, lactate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase and G-6PDH, glc-6-phosphate dehydrogenase

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Wrigglesworth, 1980; Kliman, Steck, 1980; Solti et al., 1981) and a similar conclusion had been reached also for aldolase (Yeltman, Harris, 1980).

Indications for complex formation between isolated rabbit muscle aldolase and glyceraldehyde-3-phosphate dehydrogenase (Ovádi et al., 1978; Patthy, Vas, 1978; Grazi, Trombetta, 1980) as well as insect muscle aldolase and triosephosphate isomerase (Gavilanes et al., 1981) were reported. It should be mentioned that no evidence was found for an interaction between isolated pig muscle glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase (Vas, Batke 1981).

We wished to investigate the interaction of the glycolytic enzymes with each other or with the membrane by making use of the molecular sieving property of the human erythrocyte membrane in slightly hypotonic media, as described earlier (Cseke et al., 1978). We showed that at 0 °C the membrane of swollen human erythrocytes behaved as a sieve for proteins, i.e. a fraction of the cell protein content was found to be squirted out into the cell free supernatant of partial lysates through the transiently opened pores of the distended membrane. This non-lytic release pattern showed that the smaller the molecular radius of a protein the higher was the amount lost to the medium. The effect was described for proteins of molecular radii between 2.4 and 3.9 nm.

The relationship between the non-lytic release of proteins and their molecular radii allows one to discriminate between uniform distribution and surface-near localization of proteins or complex formation with each other or the membrane. The release pattern published suggested the uniform intracellular distribution of hemoglobin and two enzymes of the pentosephosphate shunt, and the surface-near localization of three glycolytic enzymes.

In the present paper evidence will be presented that the molecular sieving phenomenon holds for proteins of smaller molecular radii too, and is independent of the effect of temperature and metabolism on the membrane resistance. The results obtained with the glycolytic enzymes studied suggest that some of them may form complexes with each other under in vivo conditions.

Materials and methods

Chemicals

Fructose-1,6-bisphosphate, 3-phosphoglycerate, D-glyceraldehyde-3-phosphate, pyruvate, glc-6-phosphate, 6-phosphogluconate, ATP, NADH, dithioerythritol, crystalline rabbit muscle triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase and α-glycerophosphate dehydrogenase were purchased from Boehringer Chem. Comp., 5,5'-dithiobis-(2-nitrobenzoic acid), acetylthiocholine iodide, acetazolamide and bovine serum albumin were Fluka preparations. All other chemicals were Reanal (Hungary) preparations of reagent grade.

Blood freshly drawn into an acid-citrate-dextrose solution pH 7.2 (cf. Cseke et al., 1978) from healthy donors of both sexes was obtained from the National Institute of Haematology and Blood Transfusion, Budapest. Following centrifugation at 900 × *g* for 15 min the plasma and the leukocytes were removed by suction and the sedimented cells were washed 3 times with an equal volume of 143 mM KCl, which contained 10 mM Na-phosphate, pH 7.5. In parallel experiments the washed sedimented cells were resuspended and incubated for 30 min *(i)* at 0 °C and 37 °C, respectively, in NaCl solutions in the absence and presence of 6 mM glucose; *(ii)* at 37 °C in the phosphate-containing washing solution in the presence of 6 mM glucose and/or 10 mM adenosine. Total osmolarity of the solutions was kept at 308 mOsm. The cells were again sedimented and partial lysis was performed as done routinely with four volumes of NaCl solutions of decreasing concentration in the absence and presence of 6 mM glucose. Following 10 min incubation at 0 °C and 37 °C, respectively, the non-lysed cells were centrifuged at 900 × *g* for 15 min. Total lysis: to 1 volume of sedimented cells 19 volumes of distilled water were added. All other manipulations were carried out as described previously.

Cell lysis is defined by the cholesterol content (cf. Cseke et al., 1978) and/or acetylcholinesterase activity (Ott et al., 1975) of total and partial lysates. Under the experimental conditions the membrane of the lysed cells remains in the cell-free supernatants. The fraction of any protein released into a partial lysate through molecular sieving was calculated as previously (Cseke et al., 1978) by subtracting its percentage derived from cell lysis from that measured in the same cell-free supernatant.

The concentration of hemoglobin (260–280 mg/ml sedimented cell) and the *activities* of the enzymes already studied were determined as previously (Cseke et al., 1978). The specific activities in total lysates, expressed in U/g hemoglobin were as follows: 3-phosphoglycerate kinase, 300 ± 25; glyceraldehyde-3-phosphate dehydrogenase, 161 ± 16; lactate dehydrogenase, 157 ± 10; 6-phosphogluconate dehydrogenase, 11 ± 1 (Cseke et al., 1978). Aldolase was assayed in the presence of fructose-1,6-bisphosphate with the coupled reaction system of triosephosphate isomerase, α-glycerophosphate dehydrogenase and NADH (Racker, 1947). If its release was tested, lysis and all dilutions were made in the presence of 2 mg/ml bovine serum albumin. Carbonic anhydrase activity was taken as the esterase activity inhibited by acetazolamide (Armstrong et al., 1966). Triosephosphate isomerase was assayed with D-glyceraldehyde-3-phosphate as substrate in the coupled reaction with NADH and α-glycerophosphate dehydrogenase (Norton et al., 1970). Acetylcholinesterase was determined according to (Ellman et al., 1961). All assay mixtures contained 0.05% Triton X-100. The specific activities in the total lysates were: aldolase, 3.75 ± 0.3; carbonic anhydrase, 11.1 ± 1.0; triosephosphate isomerase, 1800 ± 140 and acetylcholinesterase, 43.8 ± 4.0 U/g haemoglobin. All measurements were carried out at 37 °C in a Varian Techtron 635 Spectrophotometer equipped with a recorder and a termostated cuvette house. K^+ concentration was determined in an IL-243 Flame Photometer, the

total lysates contained 315 ± 16 mEq K^+/g hemoglobin. If K^+ release was measured the cell washing solutions contained only Na ions.

Molecular weigths $\times 10^{-3}$ for erythrocyte enzymes were taken as follows: carbonic anhydrase, 30 (Peterman, Hakala, 1942); 3-phosphoglycerate kinase, 49.6 (Yoshida, Watanabe, 1972); triosephosphate isomerase, 56 (Rozacky et al., 1971); hemoglobin, 68 (Braunitzer et al., 1964); 6-phosphogluconate dehydrogenase, 104 (Pearse, Rosemeyer, 1974); lactate dehydrogenase, rabbit muscle enzyme, 140 (Jaenicke, Knof, 1968); glyceraldehyde-3-phosphate dehydrogenase, 145 (Volny et al., 1968); aldolase, 158 (Yeltman, Harris, 1980) and glc-6-phosphate dehydrogenase, 210 (Cohen, Rosemeyer, 1969).

The number of active enzyme molecules per cell was calculated from data in the literature since the specific activities of the isolated enzymes were determined at temperatures different from our measurements. The specific activities of the enzymes in the total lysates (U/g haemoglobin) the specific activities of the isolated enzymes (U/mg enzyme) and the molecular weights were used for calculations. The volume of the erythrocyte was taken as 91 μm^3 (Lenard, 1974) and the haemoglobin content of 1 ml of packed red cell as 330 mg.

The relationship between the non-lytic release of proteins and their molecular radii was calculated according to Eq. 1, as described previously. The relationship holds if a practically unique pore radius is assumed (Cseke et al., 1978).

$$\frac{C_i^{\text{non-lytic}}}{C_i^{\text{total}}} = k(r - r_i)^2\pi \qquad (1)$$

where $C_i^{\text{non-lytic}}$ is the concentration of protein *i* released through molecular sieving, C_i^{total} is its concentration in the total lysate and r_i is the molecular radius. Constant k is a function of the osmotic pressure difference, r is the radius of the transiently opened pores in the distended membrane of swollen cells. Molecular radii were calculated from molecular weights and the specific volumes were taken as 0.73 ml/g (Tanford, 1961).

Computations were carried out by using a HP-9825 A programmable desk calculator.

Results

Membrane resistance of erythrocytes

Washed erythrocytes were incubated for 10 min with NaCl solutions of decreasing concentration in the absence and presence of 6 mM glucose at 0 °C and 37 °C. Figure 1 shows the *real* cell disruption as defined in Methods (not conventional "haemolysis") as a function of initial external osmolarity for experiments performed under the above conditions. In each experiment blood samples drawn from the same donor were compared. At 0 °C, under the experimental conditions inducing lysis, human erythrocytes began to disrupt around and external osmolarity of 160 mOsm both in the absence and presence of added glucose (Curve C). At 37 °C both in the absence and presence of glucose lysis began at a lower osmo-

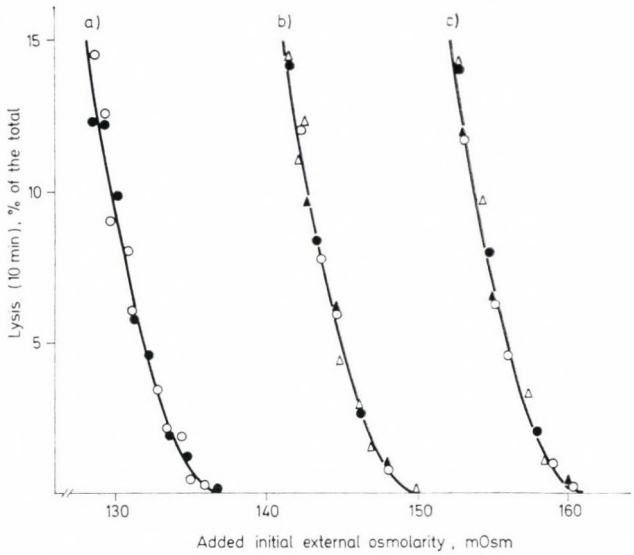

Fig. 1. Human erythrocyte lysis by decreasing external osmolarity. Single experiments were performed at 0 °C and 37 °C with blood samples obtained from the same donors. Preincubation and partial lysis were carried out in the absence and presence of 6 mM glucose, Curve a, 37 °C, samples preincubated with 10 mM adenosine; Curve b, 37 °C; Curve c, 0 °C. Cholesterol (△) and acetylcholinesterase (○). Full symbols represent the presence of added glucose. The points shown are average values of 11 experiments. For the fit of the data cf. text. The lysis of damaged cells was substracted (cf. Fig. 2a and d)

larity and the curve was further shifted toward hypotonicity if the cells were preincubated with adenosine (Curves b and a). The shift in lysis to lower osmolarities at 37 °C reflects an increased membrane resistance most probably due to the higher membrane fluidity and/or preserved membrane elasticity. However, the membrane is even more resistant to hypotonicity if ATP formation is stimulated by preincubation of the cells with adenosine. These results are in agreement with other red cell membrane phenomena influenced by cellular ATP level (Nakao et al., 1960; Gárdos et al., 1966).

It is worth noting that the data of all lysis vs. osmolarity curves in the range tested could be fitted to the equation $y = 0.193(x - b)^2$ differing in the b value. The b value reflects the differences in the membrane resistance expressed in osmolarity units. Depending on blood samples b varied in the range of ± 3 mOsm. Correlation of the experimental points and the equation varied between 0.905 and 0.915; the results obtained in 63 other experiments performed only at 0 °C also fell in this range.

Time course of the molecular sieving phenomenon

To study the time course of partial lysis of cells pretreated under different conditions the experiments were devised in such a way that treatment with hypo-

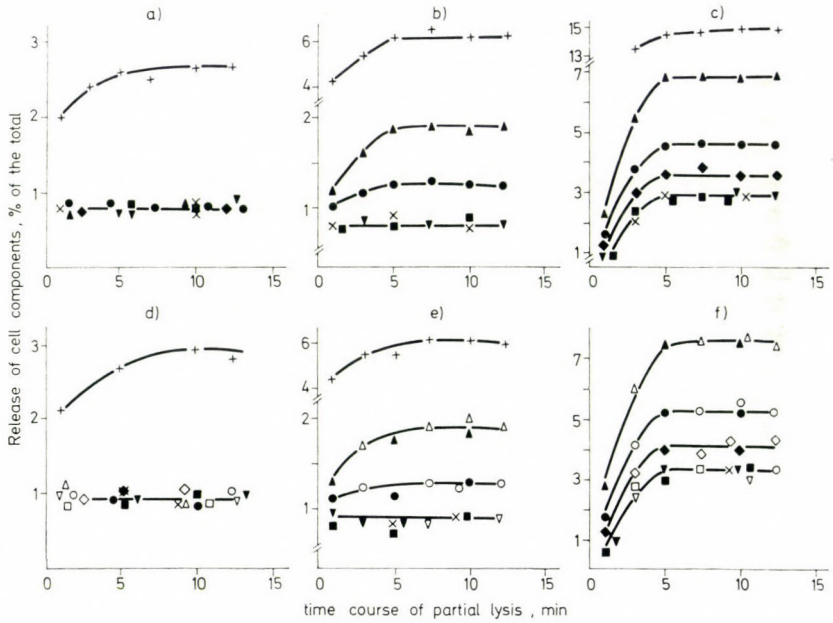

Fig. 2. Typical time course of partial lysis at 0 °C and 37 °C. The experiment was performed with blood sample drawn from a single donor. a, b and c lysis at 0 °C. d, e and f lysis at 37 °C without or with preincubation with 10 mM adenosine. Initial external osmolarities: a, b and c: 168, 164.3 and 158 mOsm, respectively. d: 156 or 143 mOsm. e: 153 or 139.5 mOsm and F: 146.6 or 133.9 mOsm. The lower osmolarity values represent the experiments with cells preincubated with adenosine (empty symbols). Cholesterol (×); acetylcholinesterase (▼) and (▽); glc-6-phosphate dehydrogenase (■) and (□); 6-phosphogluconate dehydrogenase (♦) and (◊); hemoglobin (●) and (○); carbonic anhydrase (▲) and (△); K^+ (+)

tonic NaCl solutions should give rise to about the same percentage of cell lysis (cf. Fig. 1). Three sets of experiments gave similar results, the lysis of damaged cells was 0.75, 0.85 and 1.2%, respectively, cf. below.

A typical time course of the appearance of different cell constituents in the cell-free supernatants of partial lysates is shown in Fig. 2. The results were the following: (*i*) down to a certain external osmolarity depending on temperature and ATP stimulation all cell constituents tested – except K^+ – were released in the same percentage, i.e. the proteins were lost to the medium only from lysed cells (Fig. 2, a and d). This was the small percentage of cell lysis we also observed earlier and referred to as deriving from cells damaged during manipulation or from the oldest fraction of the cell population assumed to be the least resistant to hypotonicity (cf. Cseke et al., 1978); (*ii*) at *lower* osmolarities than in a and d (b, c and e, f in Fig. 2) already after one minute the cell-free supernatants contain increasing fractions of proteins of non-lytic origin, i.e. most curves run above that of cholesterol. Acetylcholinesterase is found in the medium in amounts directly

proportional to cholesterol as expected for a membrane bound protein (Ott et al., 1975). Glc-6-phosphate dehydrogenase is also released only from lysed cells as found earlier (Cseke et al., 1978); (*iii*) the process is practically finished within 5 min when the cell content squirted out reduces the osmotic pressure difference and impermeability is restored.

K^+ is released before proteins and in much higher proportions than the proteins at any hypotonicity tested. A similar phenomenon was found for the release of K^+ (MacGregor, Tobias, 1972) and even for NADP from swollen human erythrocytes (Rácz et al., 1979).

The time course of the protein release pattern corroborates our previous results obtained after 10 min incubation of sedimented cells with NaCl solutions of decreasing osmolarity at 0 °C: the smaller the molecular weight of a protein, the higher is the percentage in which it is lost to the medium. Figure 2 also shows that this phenomenon is independent of differences in membrane resistance, since similar results were obtained at 0 °C and 37 °C and even with cells preincubated with adenosine.

Predicted localization and complex formation among some proteins within the cell

In our previous work (Cseke et al., 1978) we proposed Eq. 1 for the quantitative description of the non-lytic release of *uniformly distributed*, free proteins in the cell, according to their size (cf. Methods). By plotting the square root of the left side of Eq. 1 vs. r_i a straight line was obtained for the points of hemoglobin and two enzymes of the pentose-phosphate shunt. The points for three enzymes of the glycolytic pathway, 3-phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase fell above the straight line, i.e. these enzymes were found in the medium in higher amounts than expected from their molecular radii. In the present study in addition to the proteins studied earlier we also examined the release of carbonic anhydrase, aldolase and triosephosphate isomerase. The experiments were performed at 0 °C and 37 °C with blood samples obtained from the same donors under the conditions described in the legends to Fig. 1.

As shown in Fig. 3 the points for the uniformly distributed proteins and that of lactate dehydrogenase exhibit the same pattern at both temperatures irrespective of the membrane resistance difference shown in Fig. 1. Carbonic anhydrase also seems to be a uniformly distributed protein. Triosephosphate isomerase was released in somewhat smaller quantities than that expected from its molecular radius at both temperatures. Aldolase was not liberated in detectable amounts at either of the temperatures.

Marked differences were observed in the release pattern of 3-phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase at 0 °C and 37 °C. The points for both proteins fell significantly above the straight line at 0 °C. However, at 37 °C the point for 3-phosphoglycerate kinase fell below the straight line and that of glyceraldehyde-3-phosphate dehydrogenase – although it remained above the line – was lower than at 0 °C.

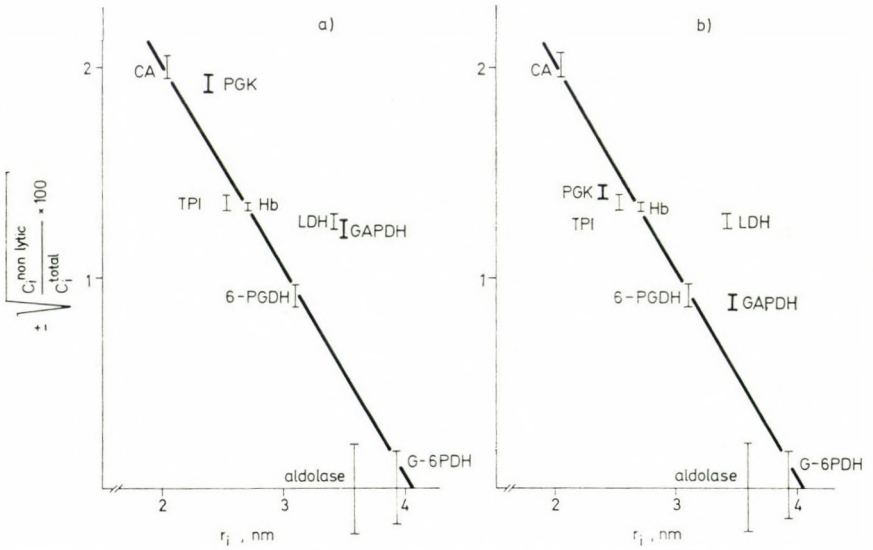

Fig. 3. Comparison of molecular sieving effect at 0 °C and 37 °C. Blood samples, treatments and calculation of lysis were the same as in the legends to Fig. 1. The basis of comparison is the same degree of lysis, viz. 2% of the total. The data are plotted according to eq. 1. (cf. Methods) and the straight lines were fitted by non-linear regression. Initial external osmolarities (mOsm): a: 0 °C, 157.8 with or without 6 mM glucose; b: 37 °C, 146.8 and 133.8 without and with preincubation with 10 mM adenosine. The bars represent standard error

It is worth noting that already at the lowest osmotic pressure difference where prelytic release is observed the proteins tested are liberated according to their size (cf. Fig. 2) and localization, and that the effective pore radii are independent of temperature and membrane resistance (Fig. 1).

Discussion

The data presented show that the molecular sieving phenomenon is not effected by the different membrane resistance of human erythrocytes under different conditions and it is already detectable within a minute of incubation in slightly hypotonic media.

If a protein is strongly bound to the membrane the point for it should fall on the abscissa in Fig. 3. On the other hand, if a fraction of a protein is complexed with the membrane or forming a complex with another protein thus being unable to pass the pore, while the rest of it is uniformly distributed within the cell, the point for it will fall below the straight line but above the abscissa. If the concentration of a particular protein is higher near the surface than elsewhere in the cell, the point for it will fall above the straight line.

The molecular sieving model on the basis of which we suggest uniform distribution, surface-near localization or complex formation among enzymes neglects charge effects. A possible charge effect would cause some problem in the interpretation of the results. Squirting out of a fluid through a pore caused by a difference in osmotic pressure is, however, a mechanical phenomenon. Therefore we assume that only gross differences in the charge of the proteins might influence this process. The available data for the isoelectric points of the proteins* do not reveal any tendency which would suggest that charge effects could account for differences in the non-lytic release pattern of proteins.

The non-lytic release pattern of proteins if tested at different temperatures showed some peculiarities with the glycolytic enzymes: the lowering in the release of glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase. The point for the latter enzyme even "changes sign", relative to the straight line of Fig. 3 at 0 °C and at 37 °C. It was also unexpected that aldolase was not liberated in detectable amounts at either temperature tested.

We propose the following explanation for the observed phenomena. Aldolase, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase, the enzymes which do not conform to uniform distribution, catalyze consecutive reactions in the glycolytic pathway. These enzymes may form complexes with each other within the cell and this complex formation may be promoted by the high rate of glycolysis at 37 °C. If there is a binary or ternary complex of aldolase and triosephosphate isomerase and/or glyceraldehyde-3--phosphate dehydrogenase, the cell should contain a minimal amount of free aldolase the release of which cannot be detected within the experimental error of our measurements. Complex formation between glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase is strongly supported by our data.

One may estimate the number of enzyme molecules per cell from scattered data in the literature. The data for aldolase and glyceraldehyde-3-phosphate dehydrogenase yield figures of 1.5 to 3.5×10^4 (Yeltman, Harris, 1980) and 1 to 2×10^5 (Maretzky et al., 1972; Kant, Steck, 1973), respectively. Thus glyceraldehyde-3-phosphate dehydrogenase is in a 5- to 10-fold excess over aldolase. From the data of Rozaczky et al. (1971) we calculated 7×10^4 molecules of triosephosphate isomerase per cell. These data favor our assumption that a binary or ternary complex of aldolase, triosephosphate isomerase and/or glyceraldehyde-3--phosphate dehydrogenase would highly reduce the concentration of free aldolase in the cell. On the other hand, the concentration of free triosephosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase would not be dramatically affected. As for 3-phosphoglycerate kinase we found such differences in the data in

* *Isoelectric points of human erythrocyte proteins:* hemoglobin, 6.95 (Rossi Fanelli, Antonini, 1958); carbonic anhydrase, ox erythrocyte, 5.3 (Peterman, Hakala, 1942); aldolase, 8.9 (Yeltman, Harris, 1980a); triosephosphate isomerase, 6.0 (Rozaczky et al., 1971); glyceraldehyde-3-phosphate dehydrogenase, 8.3 (Maretzky et al., 1974) and 3-phosphoglycerate kinase 7.9 (Yoshida, Watanabe, 1972).

the literature which do not allow any estimation. From the data of Yoshida and Watanabe (1972) we obtained a figure of 7.7×10^4 molecules per cell, whereas from those of De Barun and Kirtley (1977) 5.5×10^3. The latter figure would be in better agreement with our rationale but neither value is in contradiction with our conclusion.

One may speculate on the unchanged high release of lactate dehydrogenase at both temperatures. Glycolysis is the single energy source of erythrocytes. According to Solti and Friedrich (1979) erythrocytes contain different NAD pools and glycolysis seems to be sustained by glyceraldehyde-3-phosphate dehydrogenase-bound NAD. A "coenzyme recycling" could be easily visualized if glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase were associated within the cell. This could be achieved through an enzyme-enzyme interaction. This phenomenon could not be detected in our experiments.

If the above considerations reflect the in vivo conditions then the relative positions of the points of glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase and perhaps lactate dehydrogenase at 0 °C are fallaciously lower than in reality. This further supports the concept of surface-near localization of these enzymes as suggested earlier (Cseke et al., 1978).

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Identification of a Fluorescent Dye-containing Peptide of Glyceraldehyde-3-phosphate Dehydrogenase

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The NAD-induced local conformational changes in the fluorescent dye-binding region of muscle glyceraldehyde-3-phosphate dehydrogenase were studied (Ovádi et al., 1982). We have isolated a dominant peptide containing the fluorescent dye from the tryptic digest of labelled dehydrogenase, and identified this labelled amino acid residue. The data indicate that fluorescein isothiocyanate can react rather specifically with tyrosyl residue 91, which is not associated with the catalytic function of the enzyme. Tyrosyl-91 modified by the fluorescent dye does not interact directly with any part of the NAD bound at the active site of glyceraldehyde-3-phosphate dehydrogenase.

Introduction

The covalent binding of the fluorescein dye, FITC to tetrameric GAPD allowed us to follow the local conformational changes what take place in the dye's environment upon the successive binding of NAD (Ovádi et al., 1982). We suggested that the conformational alterations are transmitted cooperatively through the contact surface of subunits without influencing the strength of subunit interactions.

In this paper we report the isolation of a peptide from the tryptic digest of labelled GAPD containing the fluorescent dye and the identification of the labelled amino acid residue. Since the steric coordinates of amino acid residues of lobster GAPD has been known and a very high degree of similarity of the primary sequences of pig and lobster enzymes has been observed (Buehner et al., 1974, Moras et al., 1975), we can estimate the position of the fluorescent dye in the three dimensional structure of the enzyme.

Materials and methods

FITC celite was a Fluka preparation. Dansyl chloride was from Calbiochem. All other chemicals were reagent-grade commercial samples from Reanal. Sephadex G-25 was a Pharmacia product, polyamide layer sheets were from BDH. For paper-electrophoresis Whatman 3MM was used.

Abbreviations: GAPD, D-glyceraldehyde-3-phosphate dehydrogenase [EC. 1.2.1.12.]; FITC, fluorescein isothiocyanate; DNS, dansyl

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Trypsin was a Merck preparation. GAPD was prepared from pig muscle and recrystallized four times (Előd i, Szörényi, 1956). The specific activity of GAPD was 400–450 kat/mol tetrameric enzyme.

The labelling of apoenzyme with FITC and the determination of the number of fluorescent dye molecules bound to the enzyme were carried out as described previously (Ovádi et al., 1982).

Separation of peptides by paper electrophoresis was conducted with a tank type electrophoresis apparatus (Savant instrument). The peptides to be fractionated were applied as a narrow uniform zone on Whatman 3MM paper. The paper was wetted with the following buffer systems: at pH 6.5 pyridine-glacial acetic acid–water; at pH 1.9 glacial acetic acid-formic acid–water. In the high voltage electrophoresis apparatus the voltage gradient was 50 and 100 V/cm.

The fluorescent peptides were detected under ultraviolet light as fluorescent bands.

Results and discussion

ApoGAPD was covalently labelled with 1.1 mol of FITC per mol of tetramer as described previously (Ovádi et al., 1978, 1982). The solution of labelled enzyme was extensively dialyzed against deionised distilled water to remove the salts and liophylized.

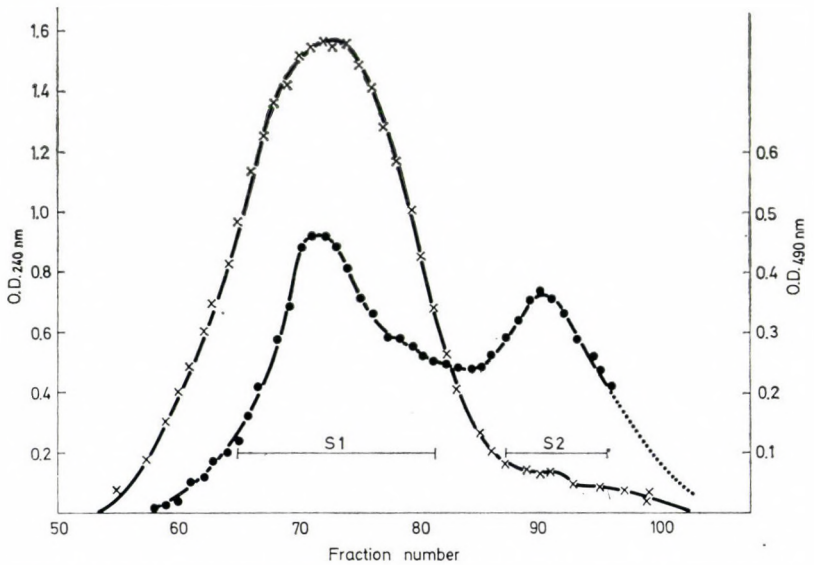

Fig. 1. Fractionation of the digested peptides. Gel-chromatographic run was made on Sephadex G-25 column (100×1.8 cm) equilibrated with 0.1 ammonium bicarbonate buffer, pH 8.5. 2 ml digest solution was loaded. Fractions (3 ml) were analyzed by absorption at 240 nm and 490 nm. Fractions from 65 to 81 and from 88 to 95 were collected as S_1 and S_2 samples, respectively

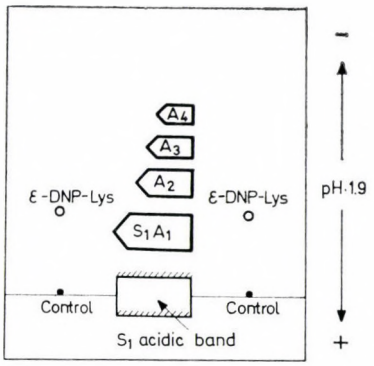

Fig. 2. Schematic picture of electrophoretogram of S_1 at pH 1.9. After electrophoresis at pH 6.5 the acidic band of S_1 was sewn to other sheet of paper and a second run was performed at pH 1.9

190 mg of dried labelled enzyme was dissolved in 19 ml ion free water, the pH of protein solution was adjusted to 8.2 by solid ammonium bicarbonate. The protein was digested by 6.4 mg trypsin for 2 hours at 37 °C and the digest was freeze dried.

The digested peptides, dissolved in 2 ml of 0.1 M ammonium bicarbonate buffer, pH 8.5, was fractionated on a Sephadex G-25 fine column (100 × 1.8 cm) equilibrated with the same buffer solution. Figure 1 shows the elution profile of peptides detected at 240 nm and 490 nm. Fractions from 65 to 81 and from 88 to 95 were pooled as S_1 and S_2 samples, respectively. These fractions showed very high fluorescence intensity under the UV lamp.

S_1 and S_2 were applied to Whatmann 3MM paper and separated by two-dimensional electrophoresis at pH 6.5 and 1.9. In the case of S_1 four fluorescent spots could be observed under UV lamp, which gave positive reaction with ninhydrin. Figure 2 shows the schematic picture of the tracing of preparative electrophoretogram. The fluorescent spot S_1A_2 which showed extremely high fluorescence intensity in comparison to the others (S_1A_1, S_1A_3, S_1A_4), was eluted with 0.05 NH_4OH, and an aliquot was dried and hydrolyzed by hydrochloride acid for analysis of amino acid composition. It should be noted that sample S_2 did not contain a significant amount of fluorescent dye bound to peptides. The fluorescent spots may be due to the traces of non-covalently bound fluorescent dye and/or the degradation of the fluorescence dye during the procedure.

A similar type of experimental series was carried out with GAPD containing 0.4 mol of fluorescent dye per tetrameric enzyme (Exp. 2).

Table 1 shows the amino acid composition of the main fluorescent peptides isolated from two different preparations. After dansylation (Gray, Hartley, 1967; Hartley, 1970) we could not find any N-dansyl derivative. According to the amino acid sequence of GAPD (Harris, Perham, 1968) we could identify the peptide

S_1A_2 with the following sequence of GAPD isolated from pig muscle:

84 95
Trp-Gly-Asp-Ala-Gly-Thr-Ala-Tyr-Val-Val-Glu-Ser (Exp. 1)

84 91
Trp-Gly-Asp-Ala-Gly-Thr-Ala-Tyr (Exp. 2)

Table 1

Amino acid composition of the isolated peptides

The yields of S_1A_2 peptide were 5—10%. The nearest integer values in parentheses mean the expected values on the basis of known sequences

	Exp. 1			Exp. 2		
	S_1A_2 nmol/analysis	l = 4.4	Nearest integer	S_1A_{23} nmol/analysis	l = 6.0	Nearest integer
Lys	0.94	0.21	—	0.20	0.03	—
Asp	4.30	0.98	1 (1)	6.20	1.03	1 (1)
Thr	4.90	1.11	1 (1)	5.47	0.91	1 (1)
Ser	4.01	0.91	1 (1)	2.60	0.47	—
Glu	5.32	1.21	1 (1)	1.00	0.17	—
Pro	1.16	0.26	—	0.80	0.13	—
Gly	8.65	1.96	2 (2)	12.80	2.12	2 (2)
Ala	6.76	1.55	1—2 (2)	12.60	2.10	2 (2)
Val	5.73	1.32	1—2 (2)			
Ile	0.81	0.18	—	0.20	0.03	—
Leu	1.74	0.39	—	0.71	0.12	—
Tyr	1.23	0.28	± (1)	1.90	0.32	± (1)
Phe	2.10	0.48	—			

The negative result of dansylation is due to the Trp N-terminus, since the dansyl derivative of tryptophan decomposes during acidic hydrolysis. Residue 83 is lysine, trypsin splits after this amino acid residue. Moreover, the splitting at residue 95 was probably due to the inherent chymotryptic activity of trypsin. The splitting after tyrosyl residue is remarkable since it is suggested that the Tyr-91 is modified by fluorescent dye. Since the two peptides are derived from the same region of GAPD we can conclude that FITC can react rather specifically.

The tyrosyl residue of the isolated peptides may be the candidate for reaction with FITC (Maeda et al., 1969). Thus the relatively low tyrosyl content of the peptide (Table 1) may be attributed to the decomposition of the FITC derivative of tyrosine during acidic hydrolysis.

On the basis of these results we suggest that the Tyr-91 of GAPD can preferentially react with FITC forming fluorescein derivative.

Tyr-91 is conserved in the amino acid sequence of pig and lobster GAPD (Harris, Perham, 1968; Davidson et al., 1967) is not involved in the active center

region or in the contact surfaces generated by P, Q and R axes (Buehner et al., 1974).

Since the coordinates of amino acid residues and of the different regions of the bound NAD of muscle GAPD are known (Moras et al., 1973) the distance of the hydroxylic group of tyrosil-91 and the adenine moiety of NAD can be calculated. We have found that the shortest distance which is between the labelled amino acid and N_1 or N_3 atoms of adenine moiety is 230 nm. Hence we can exclude the possibility of any direct interaction of the moieties of FITC and NAD. Therefore, we can conclude that the NAD induced conformational changes (Ovádi et al., 1982) are transmitted through the protein fabric and manifest themselves in the region near the tyrosil-91 located in the NAD binding domain (Rossmann et al., 1973), but not associated with the catalytic function of the enzyme.

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Homologous Partial Sequences in Dehydrogenases

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Such details of the primary structure were sought that are common in all dehydrogenases of known amino acid sequence. Twenty-six sequences of eight kinds of dehydrogenase (D-glyceraldehyde-3-phosphate dehydrogenase, alcohol dehydrogenase, lactate dehydrogenase, glutamate dehydrogenase, glycerol-3-phosphate dehydrogenase, ribitol dehydrogenase, L-hydroxyacyl-CoA dehydrogenase and homoserine dehydrogenase) have been compared by the aid of the artificial intelligence language Prolog, the amino acids being classified into groups according to their chemical properties, and alpha-helix or beta-sheet-forming abilities. We found tetrapeptides that occurred in all dehydrogenases examined. By using these tetrapeptides as markers a population of 84 partial sequences has been described. The partial sequences constituting this population are peptides comprising 35 residues.

It has been shown statistically that these peptides form a homogeneous sample as regards the frequency of occurrence of amino acid groups. This statistically homogeneous partial sequences can be regarded as homologous and it is assumed that their presence is characteristic of dehydrogenases.

Introduction

Numerous methods are available for the investigation of homologies, structural and functional similarities, as well as evolutionary relations, within various groups of enzymes. When the primary structure of enzymes is being studied from this viewpoint the underlying rationale is always the hypothesis that like functions are determined by like structures.

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Abbreviations used: ADH — alcohol dehydrogenase; B — bovine; C — chicken; CH — chicken heart muscle, CM — chicken skeletal muscle; CoA-DH — L-3-hydroxyacyl-CoA dehydrogenase; DM — Drosophila melanogaster; FM — dogfish skeletal muscle; GAPD — D-glyceraldehyde-3-phosphate dehydrogenase; GDH — glycerol-3-phosphate dehydrogenase; GlDH — glutamate dehydrogenase; H — human; HDH — aspartokinase-I-homoserine dehydrogenase; HL — horse liver; L — lobster; LDH — lactate dehydrogenase; MT — mouse testis; P — pig; PH — pig heart muscle; PM — pig skeletal muscle; RDH — ribitol dehydrogenase; RL — rat liver; S — Bacillus stearothermophilus; T — Thermus aquaticus; Y — yeast

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It is known that the tertiary structure which secures biological function is more conservative than the primary structure (Rao, Rossmann, 1973; Ohlsson et al., 1974; Eventoff, Rossmann, 1976; Argos et al., 1978; Rossmann et al., 1974, 1977). However, these sterically neighbouring elements have been properly positioned during the course of evolution of primary structure. It can thus be assumed that functional homology is also reflected at the level of primary structure.

Smith (1966) when analysing the amino acid composition found no correlation with function. By the aid of an index based upon amino acid composition (Metzger et al. 1968), Fondy, Holohan (1971) and Moor, Goodman (1977) were able to characterize quantitatively the homology of dehydrogenases. Similar comparisons are rendered possible also by other indices and coefficients (Holmquist et al., 1972; Harris, Teller, 1973; Dedman et al., 1974; Vogel, 1975; Black, Harkins, 1977).

The coenzyme-binding and catalytic domains of dehydrogenases are of very similar three-dimensional structure (Rossmann et al., 1975; Eventoff, Rossmann, 1975; Rossmann, Argos, 1976; Argos et al., 1978). Salicylates (Dawkins et al., 1967) and quinaldate (König, 1975; König et al., 1975) have been shown to be universal inhibitors of dehydrogenases. These data suggest that dehydrogenases may also have something in common at the level of primary structure. This assumption is supported by the idea that the regulatory center postulated by Ovádi et al. (1972) in D-glyceraldehyde-3-phosphate dehydrogenase is a domain produced by gene-duplication in GAPD (Engel, 1973a, 1973b), which occurs twice also in glutamate dehydrogenase. Smith et al. (1970) have shown that a 12-membered peptide, whereas Eventoff, Rossmann (1975) pointed out that a 38-membered peptide, in GlDH is remarkably similar to a partial sequence in GAPD. The sequence homology around the reactive amino acid side chains in dehydrogenases is also well documented (Harris, Waters, 1976; Keleti, 1970).

We have raised the question whether there is a part of primary structure which at least as regards its composition, is the same in all dehydrogenases known so far. Such a similar peptide may be assigned to the function of dehydrogenation, independently of the various binding sites specific for the different substrates, or it may refer back to a common ancestor, or may be a sign of similar steric structure. In search for such similar sequence element(s) we have compared all dehydrogenases with known primary structure, the amino acids being grouped into classes.

Materials and methods

The information that refers in the sequence to a particular property has to be screened from an environment of relatively high noise level. Klapper (1977) concluded from the investigation of 235 sequences at the amino acid level that statistical methods obscure the relationships inherent in the primary structure. For this reason we compared dehydrogenase sequences classifying amino acids into various groups. The classification adopted by us is based upon the data of Day-

hoff (1972), which take into account the chemical properties of amino acids as well as the accepted point mutations. In the formation of groups we have considered the work of Rekker (1977), Bull, Breese (1974) and Nozaki, Tanford (1971), who calculated or measured the hydrophobicity of amino acids, as well as the categories of Manavalan, Ponnuswamy (1977, 1978) who calculated hydrophobicity of amino acids in proteins of known sequence.

Taken all the above aspects together, we set up the following six groups:

1 – Cys
2 – His, Arg, Lys
3 – Ile, Leu, Met, Phe, Trp, Tyr, Val
4 – Ser, Thr
5 – Ala, Asn, Gln, Gly, Pro
6 – Asp, Glu

It is noteworthy that in the comparison of sequences of lobster, pig muscle and yeast GAPD's (Olsen et al., 1975) the observed amino acid replacements show good correlation with the above classification according to chemical nature.

The amino acids were also classified according to Chou, Fasman (1978) on the basis of both α-helix-former and β-sheet-former abilities.

The comparison was carried out with the following 26 known polypeptide sequences of 8 types of dehydrogenases:

1. *D-glyceraldehyde-3-phosphate dehydrogenase* (EC 1.2.1.12) from pig muscle (Harris, Perham, 1968), lobster muscle (Davidson et al., 1967), yeast (Jones, Harris, 1972), *Thermus aquaticus* (Hocking, Harris, 1980), *Bacillus stearothermophilus* (Walker et al., 1980).

2. *Glutamate dehydrogenase* (EC 1.4.1.3) from chicken muscle (Moon et al., 1972; Smith, 1979), bovine muscle (Moon et al., 1972; Moon, Smith, 1973; Julliard, Smith, 1979), human muscle (Julliard, Smith, 1979; Smith 1979), NAD-specific enzyme from *Neurospora crassa* (EC 1.4.1.2, NAD-GlDH, Austen et al., 1977), NADP-specific enzyme from *Neurospora crassa* (EC 1.4.1.4, NADP-GlDH, Wootton et al. 1974).

3. *Alcohol dehydrogenase* (EC 1.1.1.1) from horse liver (Jörnvall, 1970a, 1970b, 1970c), rat liver (partial sequence, Eklund et al., 1976a), yeast (Jörnvall, 1977a), fruitfly Drosophila melanogaster alleloenzymes (DM-ADHs, DM-ADHf, DM-ADHuf, Thatcher, 1980).

4. *Lactate dehydrogenase* (EC 1.1.1.27) from dogfish skeletal muscle (Taylor et al., 1973), chicken skeletal muscle and chicken heart muscle (Kiltz et al., 1977), pig skeletal muscle and pig heart muscle (Torff et al., 1977), mouse testis (Pan et al., 1980).

5. *Glycerol-3-phosphate dehydrogenase* (EC 1.1.1.8) from rabbit muscle, (Otto et al., 1980).

6. *Ribitol dehydrogenase* (EC 1.1.1.56) from Klebsiella aerogenes (Morris et al., 1974).

7. *Aspartokinase-I-homoserine dehydrogenase* (EC 1.1.1.3) from E. coli K 12, partial sequence (Sibilli, LeBras, 1979).

8. *L-3-Hydroxyacyl-CoA dehydrogenase* (EC 1.1.1.35) from pig heart muscle (Bitar et al., 1980)

The sequences were also checked from the atlas compilations (Dayhoff, 1972, 1973, 1976, 1978; Croft, 1973).

Such octapeptides were sought which independently of their location within the enzyme, differed from each other only at no more than two positions independently of the position of differences. If more rigorous criteria are adopted, reasonable similarities may be lost, whereas in the opposite case an overwhelming amount of data is obtained, which obviates evaluation.

Since the sequences of enzymes can be regarded as linear graphs, it became possible to employ an artificial intelligence language, the PROLOG. This language, by virtue of its ability to generate algorithms and to match pattern, is suitable for the appropriate organization of graph-handling procedures. The PROLOG works on the basis of a theorem-proving procedure of deductive mechanism which, in turn, is founded on Horn-clauses (Roussel, 1975; Warren, 1976; Clocksin, Mellish 1981).

The sequences of dehydrogenases were compared pairwise. In the pairwise similar octapeptides one pentapeptide, sequentially identical at the level of group properties, or at least its first or second tetrapeptide could be found. The pentapeptide thus obtained was "23356", accordingly the first tetrapeptide was basic in character: "2335" whilst the second was acidic: "3356". These tetrapeptides are encountered several times in all dehydrogenases examined. Therefore, we used the tetrapeptides found as markers in a search for similar partial sequences in the dehydrogenases.

It was questionable how large partial sequences are to be compared. There were hints in the literature that amino acid sequences may be regarded as ergodic systems (Misra, 1978; Yockey, 1974; Cullmann et al., 1970). There is a correlation between the amino acid in question and the composition of its environment (Krzywicky, Slonimski, 1967; Laird, Holmquist, 1975; Holmquist, 1975; 1978). Such correlation can also be observed if the amino acids are grouped according to their chemical properties (Morgan, 1960). Black et al. (1976) and Fleming et al. (1972) found that a given amino acid could influence its environment at a maximum of 10 to 14 residue distance toward both the N- and C-termini. In the first approximation, we chose the partial sequences comprising the marker tetrapeptides so that they contained, in addition to the markers, 20–21 residues toward the N- and C-termini. Thus the sequences dealt with in this analysis will be referred to as 45-membered peptides.

Statistical investigations

In order to check whether the tetrapeptides found by us are in an ergodic relation with their environment in the sequence, we performed the independence

analysis, by the aid of the non-parametric G-test (Sokal, Rohlf, 1969), of the partial sequences containing the above tetrapeptides, by classifying the amino acids into groups according to their chemical properties.

The sample was regarded as homogeneous if the distribution of occurrence of amino acid groups was similar in the sequences examined.

The significance level was determined by the aid of standard normal distribution. This was feasible, since the G-distribution well approximates the khi-square distribution if the number of samples is great. From the latter, in turn, an approximately standard normal distribution can be derived by suitable transformation (Wilson, Hilferty, 1931).

Our null-hypothesis was that the frequency of occurrence of amino acid groups is independent of the origin of sequences studied, i.e. it is not characteristic of the individual enzymes but rather in general they are similar in the data base examined. If the G-test shows significance at $p < 95\%$ level, the null-hypothesis is discarded. In order for independence to be significant, $p \geq 95\%$ should hold. In this instance we say that the level of significance of homogeneity is 5%. Independence is regarded as strongly significant (i.e. the sample is highly homogeneous), if $p \geq 99\%$ (in the following: homogeneity of 1% significance level).

Inasmuch as the sample comprising partial sequences derived from various dehydrogenases according to the G-test proved to be significantly or strongly significantly homogeneous, the partial sequences are regarded as homologous among one another. In the following homologous sequences will always be mentioned in the above sense. Although this mode of comparison of partial sequences found by us takes into account only the amino acid composition, in the case of such short peptides this is justifiable, and homology can be proved or disproved with appropriate certainty (Cornish-Bowden, 1977, 1978, 1979; van Druten et al., 1978). In the case of large polypeptides or proteins this method is, of course, of limited value (Klapper, 1977; Woodward, 1978).

Based on the G-test we worked out a computer algorithm by the aid of which the individual sequences can be omitted in such an order that the remaining ones should be the least inhomogeneous. Similar analyses were carried out also regarding the size of partial sequences. This was done to decide whether the arbitrarily chosen environment encompassing 20–21 residues was indeed necessary toward the N- and C-termini.

The PROLOG program system was written for the Siemens 4004 type computer of the Inst. for Coordination of Computer Techniques, Budapest. All other calculations were made with a HP 9825A type desk-top calculator.

Results and discussion

The 45-membered sequence containing the tetrapeptides found in dehydrogenases are listed in Table 1. In the following we concern ourselves either with the complete sequences of enzymes or with the data base of this table.

Table 1

Initial data of homogeneity investigations

In the table the 45-membered sequences containing the basic tetrapeptide (b), the pentapeptide (ba) and finally the acidic tetrapeptide (a) are presented. Partial sequences that in some tests deteriorated homogeneity are marked with an asterisk. R denotes partial sequences omitted as redundant in the studies indicated in the text. The number after the enzyme name denotes the position in the linear amino acid sequence where the marker tetra- or pentapeptide starts. Some of the sequences are peptides close to the N- or C-terminus, and therefore contain less than 45 residues

b			ba			a		
						R	P-GAPD, 73	S-GAPD, 58
							L-GAPD, 72	S-GAPD, 218
					P-CoA-DH, 113	R	L-GAPD, 282	S-GAPD, 243
*	T-GAPD, 305		GDH, 19		P-CoA-DH, 288		Y-GAPD, 283	T-GAPD, 28
R	DM-ADHf, 206		GDH, 61	R	P-GAPD, 216		H-GlDH, 371	
R	DM-ADHs, 206		RDH, 133	R	L-GAPD, 215		H-GlDH, 182	
	DM-ADHuf, 206		MT-LDH, 154		Y-GAPD, 216	R	B-GlDH, 178	
	Y-ADH, 318		PH-LDH, 6	*	Y-GAPD, 159	R	B-GlDH, 367	
	Y-ADH, 196		PH-LDH, 76	R	RL-ADH, 331	R	C-GlDH, 370	
*	Y-ADH, 171		PH-LDH, 157		HL-ADH, 330	R	C-GlDH, 181	
*	Y-ADH, 91	R	PM-LDH, 156		H-GlDH, 495		NADP-GlDH, 135	
R	RL-ADH, 39	R	CM-LDH, 156		H-GlDH, 346		NAD-GlDH, 136	
R	RL-ADH, 88	R	CH-LDH, 156	R	B-GlDH, 342		NAD-GlDH, 603	
R	RL-ADH, 219		FM-LDH, 158	R	B-GlDH, 491		NAD-GlDH, 655	
R	* RL-ADH, 349		FM-LDH, 6	R	C-GlDH, 493	*	HDH, 60	
	HL-ADH, 348			R	C-GlDH, 345		GDH, 134	
	HL-ADH, 218			*	NADP-GlDH, 330		RDH, 152	
	HL-ADH, 139				HDH, 19		MT-LDH, 10	
	HL-ADH, 88			*	RDH, 53		MT-LDH, 170	
	HL-ADH, 39			R	CM-LDH, 171		MT-LDH, 186	
	NADP-GlDH, 44			R	CH-LDH, 75		PH-LDH, 189	
	NADP-GlDH, 92						PH-LDH, 173	
	NAD-GlDH, 72					R	PM-LDH, 172	
	NAD-GlDH, 109					R	PM-LDH, 188	
						R	CH-LDH, 188	
						R	CH-LDH, 172	
							FM-LDH, 174	
							FM-LDH, 292	

Table 2

Relative frequency of occurrence of amino acid groups

	Dehydrogenases			Proteins in general*	
amino acid group	total sequences	45-membered peptides of Table 1	35 membered peptides without redundant sequences	Dayhoff et al. (1978)**	Klapper (1977)**
1	0.015	0.018	0.018	0.029	0.028
2	0.137	0.137	0.130	0.135	0.137
3	0.337	0.333	0.347	0.285	0.286
4	0.120	0.109	0.109	0.131	0.131
5	0.283	0.299	0.292	0.304	0.302
6	0.108	0.104	0.104	0.116	0.116

* Data of almost 300 proteins out of which dehydrogenase is about 5%.

** Sum of relative frequency of occurrence of amino acids belonging to the individual groups

1. *Statistical investigations*

a) *Descriptive statistical characterization.* The 26 dehydrogenases examined contained 9119 amino acids. The relative frequency of occurrence of the six groups of amino acids set up on the basis of chemical properties is summarized in Table 2. Relative frequencies were calculated by dividing the number of the given amino acid group in the examined sample with the number of all amino acids. For the sake of comparison, the relative frequencies reported by Klapper (1977) and Dayhoff et al., (1978) for the amino acids are also indicated. The latters were calculated from the sequences of a very great number of various enzymes and nonenzymic proteins.

It is seen that the ratio of Cys in dehydrogenases is close to one-half of that found in proteins in general. In addition, the ratio of hydrophobic and hydrophilic amino acids differ markedly. In the data of literature (cf. Table 2) the 3/5 ratio is about 1.07 whereas in dehydrogenases it is 0.84. It cannot be excluded that this fact reflects the abundance of oligomers among dehydrogenases relative to the data base of the literature (cf. Simon, Asbóth, 1980), or the distribution of "inner" and "outer" residues characteristic of dehydrogenases (Go, Miyazawa, 1980).

b) *Analysis of the homogeneity of similar partial sequences.* The assumed ergodic relation of tetrapeptides with their environment can only be verified if this relation is statistically identical in each of the partial sequences comprising them. It follows that by the homogeneity (or independence) test it can be simultaneously decided whether the tetrapeptides are in ergodic connection with their environment and that the partial sequences examined are of similar composition.

The whole sample (84 pieces of 45-membered peptides) when analyzed as such, gives a homogeneity of 5% significance level if 1 sequence unit is omitted. If additional two sequence units are also omitted homogeneity improves to 1% significance level (Table 3, No. 1). If only the C-terminal environment of the whole sample is preserved (20–21 membered peptides), then homogeneity is strongly significant without the necessity of leaving out any of the sequences. On the other hand, the environment on the N-terminal side, again consisting of 20 residues, allows, a homogeneity only at the 5% level. Since Simon (1979) has shown that the N-terminal part of proteins is more conservative, this might mean that after the acquisition of a sufficiently large number of point mutations an equilibrium

Table 3

Partial sequences to be omitted from Table 1 to achieve homogeneous sample

No.	Sample	Partial sequences to be omitted to achieve homogeneity at significance level of	
		5%	1%
1	Whole sample (84 45-membered peptides)	T-GAPD, 305*	RL-ADH, 349* NADP-G1DH, 330
2	Whole sample with restricted environment (84 35-membered peptides)	none	T-GAPD, 305*
3	Sample after omission of redundant sequences, with restricted environment (56 35-membered peptides)	T-GAPD, 305*	Y-ADH, 171 RDH, 53
4	Partial cluster containing "3356" type tetrapeptide, with restricted environment (51 35-membered peptides)	none	none
5	Partial cluster containing "2335" type tetrapeptide, with restricted environment (52 35-membered peptides)	T-GAPD, 305* RL-ADH, 349* RDH, 53	Y-ADH, 171
6	Partial cluster containing the pentapeptide, with restricted environment (19 35-membered peptides)	RDH, 53 NADH-G1DH, 330 Y-GAPD, 159	HDH, 19
7	Partial cluster containing "3356" type tetrapeptide, without redundant sequences, with restricted environment (32 35-membered peptides)	RDH, 53	HDH, 19 NADP-G1DH, 330
8	Partial cluster containing "2335" type tetrapeptide, without redundant sequences, with restricted environment (34 35-membered peptides)	T-GAPD, 305* Y-ADH, 91 RDH, 53 Y-ADH, 171	HDH, 19 NADP-G1DH, 330

* Sequence incomplete (1 or 2 unknown amino acids).

composition is achieved which is independent of the initial composition. Naturally, it cannot be excluded that the above tenet is only valid for complete proteins or domains, but not for small fragments of sequences. By choosing any other environment from our data base (e.g. 45-membered peptides to the right, or to the left, of those given in Table 1), homogeneity could not be achieved unless more than 50% of the sample was omitted.

The greatest improvement in homogeneity including the whole sample was attained when the environment covered 13 amino acids toward the N-terminus and 17 amino acids toward the C-terminus. Under these conditions the sample is homogeneous at 5% and the omission of a single sequence yielded 1% significance level as regards homogeneity (Table 3, No. 2). We also performed the homogeneity test by classifying amino acids into 7 groups, the 7th group being the aromatic residues (Phe, Trp, Tyr) out of the hydrophobic ones. The sample remained homogeneous also in this case.

The question arose whether the homogeneity of sample was due to the fact that of almost all sequences several nearly identical ones were considered (GAPD's, LDH's. ADH's and GlDH's isolated from different species). For this reason the enzymes isolated from different sources and having practically identical sequences were omitted. The omitted 28 redundant sequences were denoted by R in Table 1.

The significance tests were then repeated with the reduced sample thus obtained (Table 3, No. 3). It is seen that by leaving out 1 sequence unit 5%, and by the omission of further 2 units 1% significance level for homogeneity can be obtained as in the case of the widest sample (Table 3, No. 1).

We have also analyzed the partial clusters of the data base. The partial cluster comprising the acidic tetrapeptide is more homogeneous than that containing the basic tetrapeptide or the pentapeptide (Table 3, Nos 4–6). Similar results were found in the sample without the redundant partial sequences and with restricted environment (Table 3, Nos 7–8). Of the pentapeptide-containing sequences after omitting the redundant ones only eight is left, hence this small number obviates the evaluation of G-test.

It can be seen that inhomogeneity is caused in almost every case by the same sequences. It should be noted that two positions of T-GAPD, 305 are still unknown and it is primarily this fact why it represents the unit that deteriorates homogeneity to the greatest extent. If we insert in the place of unknown amino acids one Cys and one amino acid from group 4 or two amino acids from group 4, T-GAPD, 305 no longer affects homogeneity unfavourably. It is also probable that RL-ADH, 349 has been placed among the homogeneity-degrading units because it has 1 unknown amino acid in its sequence.

On the basis of the above analysis, the composition of the typical homologous 35-membered sequence is as follows (cf. Table 2): $1_{0.6}$, $2_{4.6}$, $3_{12.2}$, $4_{3.7}$, $5_{10.3}$, $6_{3.6}$.

The statistical analysis of the sequence units in Table 1 has also been performed on the basis of the beta-sheet-forming and alpha-helix-forming parameters of the amino acids as introduced by Chou, Fasman (1978). Taken the whole sample and the environment (84 pieces of 45-membered peptides) it gives a homo-

geneity of 5% significance level and only the sequences T-GAPD, 305 and MT-LDH, 10 had to be omitted for 1% significance level of homogeneity to be attained if the amino acids are grouped from the viewpoint of beta-sheet formation. Obviously, this does not mean that these structures are necessarily beta-sheets, since beta-sheet-breakers occur among them with the same weight as the beta-sheet-formers.

When the amino acids are grouped according to alpha-helix formation, for a homogeneity of 5% significance level 8 sequence units have to be omitted, whereas 11 are to be excluded for strongly significant homogeneity. If 13 and 15 amino acids are left of the environment toward the N- and C-terminals, the number of sequences to be omitted changes to 5 and 8, respectively. This indicates that our sample is not homogeneous in respect of the alpha-helix-forming ability of amino acids.

From the striking difference of the results obtained on the basis of the two types of classification it can be inferred that the beta-structure-forming property is related to the chemical features adopted by us for the classification of amino acids (cf. Darvas et al., 1980), whilst there is no such correlation with the alpha-helix-forming ability.

It seems that within the virtually homogeneous sample the distribution of occurrence of amino acids constituting the sequence units is identical statistically at the level of chemical group properties (or beta-sheet-forming ability). This may suggest the conservative nature of physico-chemical parameters. The significance of this is corroborated by the fact that enzymes tested (altogether 15 sequences, after the omission of redundant and partial sequences) were significantly heterogeneous as regards their total sequence at the level of both chemical group-properties and beta-sheet formation and more than 70% of the total sequences had to be omitted in both instances to achieve a homogeneity of 5% significance level.

The already recognized genetic relationships (Rao, Rossmann 1973; Rossmann et al., 1974, 1975) among the dehydrogenaəss could not be detected by this method, which implies that evolutionary effects tend not to manifest themselves at the level of chemical properties.

2. Location of homologous sequence units in the various dehydrogenases

a) *Glyceraldehyde-3-phosphate dehydrogenase.* Among Y-, L- and P-GAPD homology at the sequence level is about 60%, whereas between T-GAPD and the former it is about 50% (Harris, Waters 1976).

The homologous partial sequences are clustered around three sites. Partly at the N-terminal side of the nucleotide-binding domain, and partly in the center and on the C-terminal portion of the catalytic domain. The sequences marked by the acidic tetrapeptide accumulate around the N- and C-terminals, whereas the partial sequences comprising the pentapeptide are located between them (Table 4).

b) *Glycerol-3-phosphate dehydrogenase.* Otto (1977) has called the attention to a sequence analogy at the pentapeptide level in the partial sequence of rat GDH, FM-LDH, PM-LDH and mitochondrial malate dehydrogenase. This

Table 4

Distribution of environments marked by the tetrapeptides over the domains

Nucleotide-binding domain						Catalytic domain					
GAPD											
1—149						150—334					
P-	L-	T-	S-	Y-		P-	L-	T-	S-	Y-	
a	a	a	a	—		ba	ba+a	b	2a	2ba+a	
LDH											
1—165						166—329					
PH-	PM-	CH-	CM-	FM-	MT-	PH-	PM-	CH-	CM-	FM-	MT-
3b	b	b+ba	b	2b	b+a	2a	a	a	ba	2a	2a
ADH											
176—318			155—291			1—175, 319—374			1—154, 292—347		
HL-	RL-	DM-	Y-			HL-	RL-	DM-	Y-		
b	b	b	2b			4b+ba	3b+ba	—	2b		

a: environment marked by acidic tetrapeptide
b: environment marked by basic tetrapeptide
ba: environment marked by the pentapeptide
The figures indicate how many of each 35-membered peptide are found in the given domain.

pentapeptide contains the "3356" type tetrapeptide found by us. In the sequence of rabbit GDH (Otto et al., 1980) we have found two basic tetrapeptides, the environment of which are homologous with the similar environment of the other dehydrogenases and also an acidic one the environment of which is probably analogue with that of the rat GDH containing the tetrapeptide "3356".

c) *Glutamate dehydrogenase.* Out of the five GlDH sequences that occur in the data base the human, bovine and chicken enzymes are almost of entirely similar sequence even at the amino acid level (Julliard, Smith, 1979). Consequently, the four homologous environments are completely equivalent in all three enzymes. Proceeding from the N- toward the C-terminus, first we find the segment marked by the acidic tetrapeptide, then the segment marked by the pentapeptide, and this order is repeated. It appears then that with enzymes isolated from vertebrates the sequence is being repeated starting from position 355 in a shortened form.

In two *Neurospora crassa* GlDH's the above symmetry, or similar to that observed in GAPD, cannot be found. Close to the N-terminal two partial sequences marked by the basic tetrapeptide are located.

d) *Lactate dehydrogenase.* Six kinds of LDH have been examined. The chicken and pig enzymes are 76% homologous at the amino acid level (Torff et al., 1977).

In the LDH's the homologous environments usually are grouped at the border of the two domains. On the N-terminal side of reactive Cys-165, in the

nucleotide-binding domain, a homologous environment marked by the basic tetrapeptide can be found, (except MT-LDH) whereas on the C-terminal side of Cys-165, in the catalytic domain a homologous environment marked by the acidic tetrapeptide is located (Table 4).

e) *Alcohol dehydrogenase.* Four ADH sequences occur in the data base. The sequences of the two liver ADH's are very similar also at the amino acid level (Eklund et al., 1976b). In the ADH's only the basic tetrapeptide can be found in both domains, the acidic one occurs only within the pentapeptide (Table 4). Yeast and Drosophila ADHs are the sole dehydrogenases in which the "3356" type acidic tetrapeptide does not occur at all, though in Y-ADH it may appear at the expense of one amino acid insertion (Y-ADH, 196).

Jörnvall et al. (1978) claim that the partial sequences starting from position 196 in the yeast enzyme and from position 218 in the liver enzyme are homologous.

f) *Other dehydrogenases.* HDH from *E. coli* K 12, in its known 79-membered partial sequence from the N-terminus, contains two tetra-, or pentapeptide-containing environments. One of these represents homologous partial sequence.

Out of the three environments of RDH from *Klebsiella aerogenes* which contains the tetrapeptide, one is not homologous. Of the other two, one contains the acidic, the other the basic tetrapeptide. The occurrence of two homologous environments lends support to the suggestion according to which in RDH gene duplication might have taken place (Inderlied, Mortlock, 1977).

In CoA-DH from pig heart muscle two homologous environments were found, each containing the pentapeptide.

General conclusions

It cannot be excluded that there are homologous areas (environments) in the dehydrogenases examined from which the tetrapeptides used as markers are missing. However, since we found, on the average, three homologous environments per enzyme, it seems improbable that many more independent and non-overlapping homologous partial sequences would exist which fell through our screening procedure.

It can be assumed that the sequence units found by us to be homologous on the basis of chemical grouping contain folding nuclei which may play a role in the formation or stabilization of characteristic and functionally important steric structure (cf. Anfinsen, 1973; Simon, 1979). The effective distance (13 to 17 amino acids) of such an environment, which by the same token can be a folding nucleus, is in good agreement with the propagation distance set forth in Wetlaufer's (1973) work (position 8 to 18).

The amino acids that participate in catalytic or other specific functions, with a few exceptions, do not fall into the homologous areas, moreover, the residues responsible for coenzyme binding are not found here either. This feature corresponds to our preliminary expectation, inasmuch as our aim was not to look for the

sequence units comprising the essential side chains that secure the specificity of the individual dehydrogenases, but rather to look for the general that is common to all dehydrogenases.

The "3356" and "2335" type tetrapeptides can also be found in other oxidoreductases such as superoxide dismutase, catalase and dihydrofolate reductase. However, in preliminary studies performed by us on 23 sequences of 12 kinds of proteases, as well as on 35 sequences of 12 kinds of other enzymes, in not a single case could we find the "3356" type tetrapeptide.

The homologous partial sequences found by us in dehydrogenases may reflect that the only non-specific, common function of dehydrogenases is dehydrogenation, or that dehydrogenases descended from a common ancestor, or that they take part in the tailoring of similar three-dimensional structures as carriers of folding nuclei. Further investigations are needed to narrow the alternatives. Nevertheless, the available data presented in this paper seem to be sufficient for the assumption that the presence of these partial sequences is characteristic of dehydrogenases.

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Isolation and Characterization of Nuclear hnRNP Complexes from Drosophila Melanogaster Tissue Culture Cells

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Fractionation on sucrose gradients of nuclear described extracts prepared from cultured Drosophila melanogaster cells by sonication of the nuclei in the presence of rat liver cytosol RNAase inhibitor revealed a complex polysome-like pattern of nuclear ribonucleoprotein complexes. The bulk of these heterogeneous ribonucleoprotein (hnRNP) complexes sedimented in the 30*S* to 80*S* zone of the sucrose gradient. According to biochemical and morphological data, the monomer particle proved to be the 45*S* hnRNP and its average diameter was found by electron microscopy to be 24–26 nm. The buoyant density of both the mono and polyparticles was about 1.4 g/cm^3, with a slight degree of heterogeneity. The proteins from different zones of the sucrose gradient were composed primarily of similar polypeptides of 47 000, 56 000, 64 000, 96 000 and 130 000 daltons. Complete dissociation of nuclear hnRNP complexes was observed by resedimentation of the particles in the presence of 0.7 M NaCl or 4 M urea. RNAase A digestion (0.1 μg/ml at 0 °C for 10 min) resulted in the solubilization of part of the hnRNP and aggregation of some particles. The bulk of the RNA isolated from the different sized hnRNP complexes sedimented in the 7 to 11*S* region in the sucrose gradient. The large hnRNP complexes contained hnRNA strands larger than 15*S*, up to 28*S*. The base composition of the RNA from the 45S monoparticles proved to be AU type: A + U/G + C = 1.7. The RNAs from the 60–75*S* and 90–100S polyparticles were also AU type, with an A + U/G + C ratio of 1.46 and 1.21, respectively. The hnRNP complexes exhibited marked heterogeneity in the electron microscope. Our biochemical and morphological observation point to a nonrandom organization of hnRNP particles in *Drosophila melanogaster* nuclei.

Introduction

In eukaryotic cells the bulk of mRNA is transcribed in the form of high-molecular-weight precursors, hnRNA. The majority of these molecules are metabolized within the nucleus very soon after their synthesis, leading to a selective

Abbreviations: hnRNP, heterogenous ribonucleoprotein; mRNA, messenger ribonucleic acid; RNP, ribonucleoprotein; RNAase, ribonuclease; SDS, sodium do decylsulphate

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preservation of the relevant messenger sequences. About 10–20% of the newly synthetized hnRNA could be found as functional mRNA on the polyribosomes (Kewin, 1975; Ryffel, 1976).

Biochemical evidence and electron microscopy of spread chromatin have indicated that the hnRNAs are associated with a set of specific proteins to form ribonucleoprotein particles (Laird, Choi, 1976; McKnight, Miller, 1979; Beyer et al., 1980, 1981). It seems likely that the events of mRNA processing, such as the capping of 5' termini, 3' polyadenylation, rearrangement, selection and transport, occur within the framework of RNP particles (Van Venrooij, Janssen, 1978; Knowler, Wilks, 1980; Jeanteur, 1981).

From these data it appears important to study the role of hnRNP complexes in the post-transcriptional processing of mRNAs. Especially in mammalian and vertebrate cells the structure and function of hnRNPs have been intensively investigated (Samarina et al., 1968; Samarina, Krichevskaya, 1981; Martin et al., 1977; Preobrazhensky, Spirin, 1978; Van Venrooij, Janssen, 1978). Only a few publications have dealt with hnRNP particles of intervertebrate cells (Firtel, Pederson, 1975; Wooley et al., 1981).

Recently (Wooley et al., 1981) the nuclear ribonucleoprotein particles from Drosophila embryos were characterized and the subunit structure was assumed to be a 30*S* RNP. From cultured Drosophila melanogaster cells, under our experimental conditions, the monoparticles proved to be 45*S* (Szabó et al., 1981).

Materials and methods

Materials

[^{3}H] Uridine (15 Ci/mmol) was purchased from the Institute for Res. Production and Application of Radioizotopes (Prague) and $K_2H^{32}PO_4$ from the Institute of Radioisotopes, Hungary. CsCl and Kenacid blue R were obtained from BDH Chemicals Ltd, SDS, EGTA, HEPES, spermine and spermidine from SERVA, 2-mercaptoethanol from Merck, ribonuclease T1 and T2 from SIGMA. All other chemicals were purchased from Reanal (Hungary). Phenol was redestilled, acrylamide and N,N'-methylene-bis-acrylamide were recrystallized before use. All buffers containing sucrose were pretreated with 0.1% diethyl-pyrocarbonate (SERVA) before use to eliminate ribonuclease contamination.

Cell culture and radioactive labelling

Drosophila melanogaster tissue culture cells (line KC 161) were grown in suspension in medium D 22 (Echalier, Ohanessian, 1970) containing 2% fetal calf serum. The cells grown in spinner flasks at 25 °C had a generation time of about 24 h and were maintained at a concentration between 2×10^6 and 5×10^6 cells/ml. For labelling, the *Drosophila* cells were concentrated 5-fold and incubated in Schneider medium in the presence of 30–50 μCi/ml 5-^{3}H uridine or 100 μCi/ml $K_2H^{32}PO_4$ (carrier-free) for 90–100 minutes.

Buffers

Buffer A: 50 mM HEPES, pH 7.2, 1 mM EDTA, 0.25 mM EGTA, 5 mM spermidine, 0.15 mM spermine, 20 mM 2-mercaptoethanol. Buffer B: 100 mM NaCl, 10 mM TEA-HCL pH 8.0, 1 mM $MgCl_2$, 5 mM 2-mercaptoethanol. Buffer C: 25% (v/v) formaldehyde, 5 mM sodium phosphate, pH 7.5. Buffer D: 8 M urea, 1% SDS, 1% 2-mercaptoethanol, 10 mM sodium phosphate, pH 7.1. Buffer E: 0.1% SDS, 5 mM sodium phosphate, pH 7.1. Buffer: F: 75 mM NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2% SDS. Buffer G: 75 mM NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.25% SDS.

Preparation of RNase inhibitor

RNAase inhibitor was prepared from rat liver cytosol according to Roth (1958). RNAase inhibitor from six livers was used for each experiment. Buffers A and B and the sucrose cushions contained the inhibitor.

Preparation of cell/nuclei and nuclear extract

The labelled and washed cells were resuspended in buffer A and frozen in liquid nitrogen. The frozen suspension was thawed and centrifuged. The pellet was homogenized successively in buffer A containing 30% w/w and 60% w/w sucrose. The nuclei were then purified by centrifugation through a 60%, w/w and 75% w/w discontinuous sucrose gradient in buffer A. Nuclei which appeared pure by electron microscopy were sonicated in buffer B (Stevenin and Jacob, 1974). The chromatin and nucleoli were pelleted and the supernatant was then used for further analysis. In our recent publication (Szabó et al., 1981) the procedure of the isolation of nuclei has been reported in detail.

RNAase treatment of hnRNP particles

The hnRNP samples were treated with 0.1 μg/ml of ribonuclease A for 10 min at 0 °C. The control was kept at 0 °C during the same time.

Centrifugation of experiments

After sonication and centrifugation the supernatant, designated as 'nuclear extract', was layered on a 15–30% (w/w) sucrose gradient in buffer B and centrifuged for 6 h at 26 000 rpm at 2 °C in an SW 27 Beckman rotor. 2–3 ml fractions of the above sucrose gradient were collected and recentrifuged under different conditions (as indicated below). Sedimentation coefficients were determined with rat liver nuclear 30*S* particles, and ribosomal subunits or 18*S* and 28*S* rRNA from rat liver as markers. The different regions of the first sucrose gradient were fixed with 4% neutralized formaldehyde and dialysed against buffer C. The dialysed materials were mixed with concentrated CsCl solution to a final density of 1.2 and 1.7 g/cm^3 in buffer C. The dyalysed materials were mixed with concentrated CsSl solution to a final density of 1.2 and 1.7 g/cm^3 in buffer C. Then a preformed density gradient was made and ultracentrifuged in a SW 41 Beckman rotor for 46 h at 35 000 rpm and 6 °C.

Polyacrylamide gel electrophoresis of proteins

After trichloroacetic acid precipitation the samples were washed with 5% trichloroacetic acid, ethanol-ether, and ether, and dried at room temperature. After solubilization in buffer D, samples were incubated for 24 h at 37 °C. Proteins were analyzed on 8% polyacrylamide gels in the presence of 0.1% SDS and 6 M area in 50 mM sodium phosphate (pH 7.1) and were cast in glass tubes (Molnár, Samarina, 1975).

Isolation and characterization of RNA

The 30–40S, 45–60*S* and 90–100*S* regions of the sucrose gradient were collected, completed to 0.5% SDS, precipitated with 2 volumes of ethanol, dissolved in 0,5 ml buffer F, then layered on a 5–20% (w/w) sucrose gradient in buffer G. Ultracentrifugation was carried out in an SW 27 rotor for 13 h at 22 000 rpm at 18 °C.

Base analyses

The base compositions of the RNAs from the RNP particles sedimenting in the 40–50*S*, 60–75*S* and 90–100*S* regions of the sucrose gradient were determined on the basis of ^{32}P incorporation. The RNA samples were digested for 5–8 h at 37 °C in 50 mM ammonium acetate buffer (pH 4.5) containing 5 U/ml T2, 1000 U/ml T1 and 50 μg/ml RNAase A. The nucleotides obtained were separated by PEU cellulose thin layer chromatography. The radioactivities of the spots were determined (Volckaert, Fiers, 1977).

Electron microscopy

The isolated nuclei were examined for purity by electron microscopy. The pelleted nuclei were fixed in ice-cold buffered glutaraldehyde (2% in 0.1 M cacodylate buffer, pH 7.4) for 1 h, followed by post-fixation in buffered 2% OsO_4 for 1 h at room temperature, at pH 7.1. The samples were dehydrated and embedded in Epon-Araldite and sectioned with an LKB ultramicrotome. The ultrathin sections were stained with uranyl acetate and lead citrate (Reynolds, 1963) and examined in a JEOL 100-C electron microscope (accelerating voltage 80 kV). Samples of the hnRNP particles from the fractions of sucrose density gradients were collected and prepared for electron microscopy as described earlier (Komáromy et al., 1975). The specimens were contrasted by platinum-palladium shadowing in a JEOL IEE-4 B vacuum evaporator. For negative staining the preparations were stained with a 1% aqueous solution of uranium acetate. The electron microscopic examinations were carried out as described above.

Results

The method commonly used to purify nuclei from cell cultures with detergents was not successful in our experiments. In some cases we also used NP 40, but this yielded contamination of pre-ribosomal particles with RNAs (not shown).

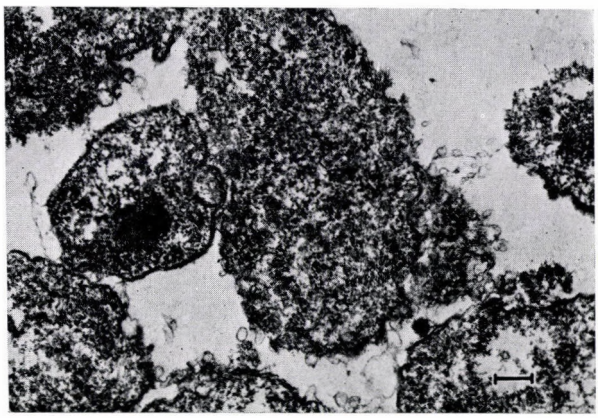

Fig. 1. Survey electron micrograph of nuclei isolated from Drosophila melanogaster tissue culture cells (line KC 161). Magnification × 20 000. Bar represents 0.5 μm

We therefore isolated the nuclei without any detergent. Instead the lysis buffer and sucrose cushions contained EDTA. EDTA can be used effectively to free nuclear membranes from ribosomal contaminants (Louis and Sekeris, 1976) (Fig. 1).

Sedimentation and re-sedimentation of ribonucleoproteins on sucrose and buoyant density gradients. Submicroscopic organization.

A [^{3}H]-Uridine labelled nuclear extract was centrifuged on a 15–30% sucrose gradient. The labelled material was sedimented as polysome-like complexes. The main part of the radioactivity appeared in the 30 to 80*S* region (Fig. 2). In order to examine the organization of hnRNP and to find the monomer particle,

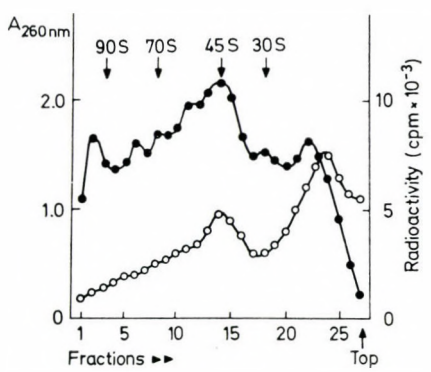

Fig. 2. Sedimentation of a nuclear extract in a 15—30% sucrose gradient. Centrifugation for 6 h at 26 000 rpm and 2°C in a Beckman SW 27 rotor. (○— ○) A_{260}, (●— ●) trichloroacetic acid precipitable radioactivity of 10 μl samples (cpm)

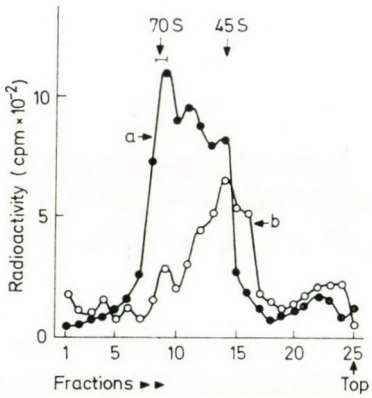

Fig. 3. Resedimentation in a 15–30% sucrose gradient of hnRNP from the 70*S* region of the first sucrose gradient (cf. Fig. 2). Centrifugation for 10 h at 22 000 rpm and 2 °C in an SW 27 Beckman rotor. Radioactivity of trichloro-acetic acid precipitable material resedimented under (●— ●) normal and (○— ○) mild autodegradation conditions 100 μl samples

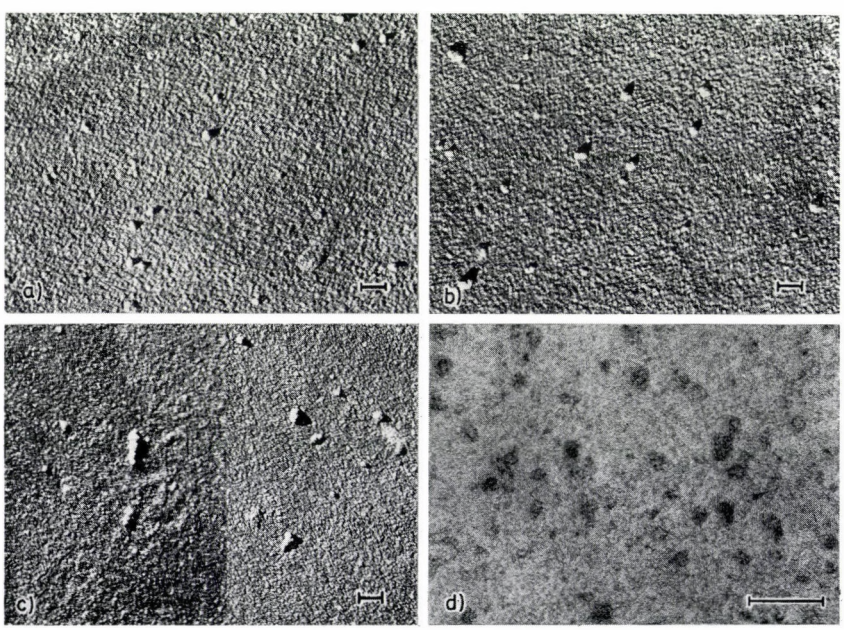

Fig. 4. Electron micrographs of hnRNP particles isolated from Drosophila melanogaster tissue culture cells. A. Monomer particles obtained from the 45*S* region of a sucrose gradient. B. Dimer particles obtained from the 60*S* region of a sucrose gradient. C. Polyparticles containing several monomers as obtained from the 90–100*S* region of a sucrose gradient. The specimens in A, B, and C were contrasted by platinum-palladium shadowing. × 64 000. Bars represent 100 nm. D. The same as A, but the samples were stained by 1% uranium acetate in water. × 200 000. Bar represents 100 nm

the 45*S* and the heavier regions (70 and 90–100*S*) of the sucrose gradient were resedimented on a second sucrose gradient. During this re-sedimentation the main part of the heavier material sedimented to the same position, but there was a shift of radioactivity up to 45*S* (Fig. 3, curve a). Under conditions of mild autodegradation (5 min at 20 °C prior to resedimentation) the bulk of the heavier material was recovered as 45*S* particles (Fig. 3, curve b). Uncontrolled endogenous ribonuclease activity during the resedimentation and mainly autodegradation, lead to a partial degradation of the hnRNA chains of the polyparticles and introduce large amounts of subunit structures. When a small fraction of material was run at 45*S* and recentrifuged on a second sucrose gradient, a peak with the same sedimentation coefficient could be observed (not shown). These biochemical findings suggested that the 45*S* RNP complex was possibly the subunit of the hnRNP polyparticles. In order to examine whether the 45*S* hnRNP complexes really comprise the subunit-structure, the fixed 45*S* particles and materials from the heavier zones of the sucrose gradient were examined by electron microscopy. They were shadowed or negatively stained with uranyl acetate. In part A of Fig. 4 there are shadowed globular monoparticles originating from the 45*S* region. Their size varies between 24–26 nm as calculated from the length of the shadows. In parts B and C of Fig. 4 dimers originating from the 60*S* and oligomers of 2–5 globular subunits from the 90–100 *S* zone can be seen. In part D of Fig. 4 negatively stained monoparticles are presented. The size distribution of the 45*S* hn RNP particles is given in a histogram (Fig. 5). The heterogeneity of the particles is indicated by the lengths of the hnRNP shadows as well.

After centrifugation of the poly- and monoparticles in the isopycnic CsCl density gradients, their densities seemed to be essentially the same ($\rho = 1.4$

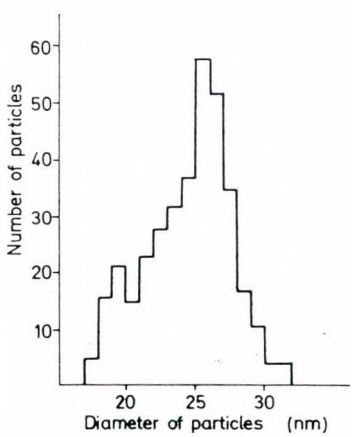

Fig. 5. Size distribution of hnRNP particles. The samples were taken from the 45*S* region of a sucrose gradient. The diameters were determined by using electron micrograph of samples shadowed by platinum-palladium and their distribution drawn in a histogram. Mean size of particles is 24.2 nm

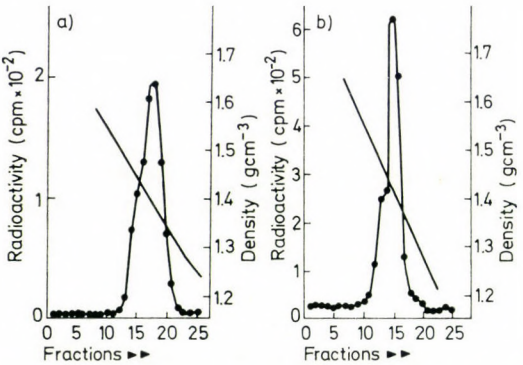

Fig. 6. CsCl density gradient centrifugation of hnRNP particles from the 45*S* (a) and from the 90 to 100*S* regions (b) of the first sucrose gradient (Fig. 2). Fixed and dialyzed material was mixed into preformed CsCl gradient with the density of 1.2 and 1.7 g/cm³ containing 2% formaldehyde in 5 mM sodium phosphate buffer (pH 7.5). Centrifugation conditions: 46 h at 35 000 rpm and 6 °C in a SW 41 Beckman rotor. (●— ●) radioactivity of 100 μl samples

g/cm³) (Figs 6a and b). A density of 1.4 g/cm³ corresponds to a protein: RNA ratio of 4 : 1 (Preobrazhensky and Spirin, 1978). A small degree of hnRNP heterogeneity, pointing to differences in RNA and protein contents, was also observed in the banding pattern.

Characterization of the hnRNP proteins by SDS-urea-polyacrylamide gel electrophoresis

The protein composition of the materials from different zones of the sucrose gradient was determined and compared with that of the 45*S* monomer. The proteins of the 45*S* particles and the heavier complexes exhibited essentially the same and very typical electrophoretic pattern (Fig. 7). It consisted of five main bands, with M_r values of 47 000, 56 000, 64 000, 96 000 and 130 000, and a few minor bands. There were no bands typical of ribosomal proteins and histones. The electrophoretic mobilities of the most intense bands are lower than those of the major polypeptides of 30*S* subparticles from rat liver nuclei (not shown.)

The RNA of hnRNP complexes

The bulk of the RNA extracted from the 30–40*S* 45–60*S* and 90–100*S* hnRNP complexes sedimented in the 7*S* to 14*S* region of the sucrose gradient (Fig. 8). The ratio and the size of the heavier RNA molecules (up to 28*S*) depended on the sedimentation coefficients of the RNP complexes from which they originated. The heavier complexes contained the longer RNA molecules.

Base analyses

The base composition of RNA from the 40–50*S*, 60–75*S* and 90–100*S* hnRNPs were determined. The hnRNA from the hnRNP particles proved to be AU type, but the ratio of A + U/G + C was different, depending on the size of

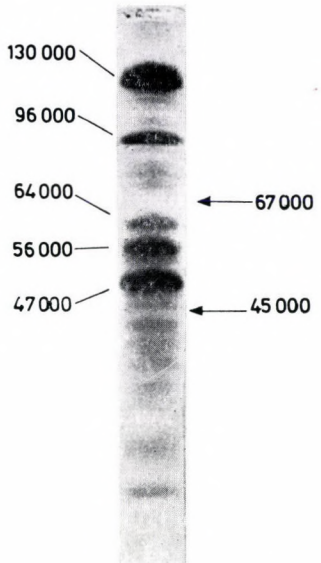

Fig. 7. Urea-SDS-polyacrylamide gel electrophoresis of the proteins of Drosophila melanogaster 50—70 hnRNP particles. The RNPs were prepared by sucrose gradient centrifugation. Electrophoresis was performed for 5 h at 7 mA per tube in 8 % polyacrylamide gels. (The M_rs of egg albumin and bovine serum albumin are indicated)

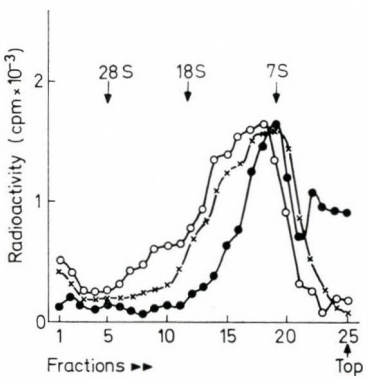

Fig. 8. Sedimentation in a 5—20 % sucrose gradient of RNAs from different-sized hnRNPs of the first sucrose gradient (Fig. 2). Centrifugation for 13 h at 22 000 rpm at 18 °C in a SW 27 Beckman rotor. Radioactivity of RNA from the (●—●) 30—40*S*, from the (×—×) 45—60*S* and from the (○—○) 60—80*S* hnRNP complexes (Trichloroacetic acid insoluble materials of 25 μl samples). The sedimentation coefficients of marker 18*S* and 28*S* rRNAs are indicated

the hnRNP. The A + U/G + C ratios obtained for the 40–50*S*, 60–75*S* and 90–100*S* hnRNP particles were 1.7, 1.46 and 1.21 (Table 1) respectively. The RNAs originating from the heavier complexes appeared to have less A + U.

Table 1

Base composition of RNAs isolated from different complexes of nuclear hnRNP

Zone of sucrose gradient	Base composition as percentage of ^{32}P radioactivity				(A + U)/(G + C)
	G	U	A	C	
40–50*S*	18.4	32.6	30.4	18.6	1.70
60–75*S*	23.2	34.3	25.2	17.3	1.46
90–100*S*	25.0	32.4	22.4	20.7	1.21

RNAs from the 40–50*S* and 60–75*S* complexes were centrifuged in a 5–20% sucrose gradient and the base compositions of the 5 to 12*S* and 12 to 20*S* RNAs were determined. The above-mentioned base distribution held true in this case, too (Table 2).

Table 2

Base composition of RNAs isolated from the 40-50S and 60-75S hnRNP complexes

hnRNP complexes	RNA from hnRNP complexes	Base composition as percentage of ^{32}P radioactivity				(A + U)/(G + C)
		G	U	A	C	
	5–12*S*	18.4	32.8	31.5	17.3	1.80
40–50*S*	12–20*S*	18.4	32.6	30.4	18.6	1.70
	5–12*S*	23.2	34.3	25.2	17.3	1.46
60–75*S*	12–20*S*	27.8	28.5	27.7	16.0	1.28

The stability of nuclear hnRNP complexes

The stability of nuclear hnRNP complexes was studied in the presence of higher salt concentrations and in urea. The 70*S* hnRNP complexes were resedimented in a 15–30% sucrose gradient containing 0.7 M NaCl. As can be shown in Fig. 9, dissociation was almost complete. A small proportion of the radioactivity sedimented in the zone heavier (20*S*) than the nascent RNAs extracted from the 70*S* particles. It appears that this may be a less salt-sensitive fraction of the hnRNP complexes (Fig. 9).

The dissociation of hnRNP complexes was more pronounced with 4 M urea (Fig. 9). The particles could be completely dissociated into RNAs and proteins of low sedimentation coefficients.

The hnRNP particles obtained from the first sucrose gradient were treated with different amounts of pancreatic RNase (in the range of 0.04 to 0.1 μg/ml). Using 0.1 μg/ml RNAase A (at 0 °C for 10 min), a significant amount of the RNA of the 45*S* complexes was degraded to low molecular weight components and aggregation of remaining particles was induced (Fig. 10).

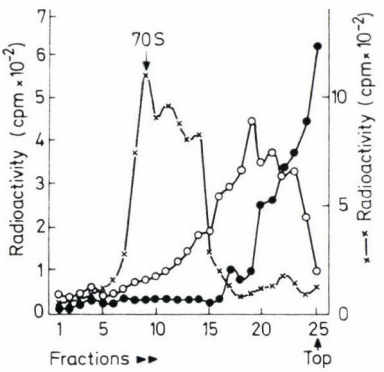

Fig. 9. Resedimentation of the 70*S* particles from the first sucrose gradient in a 15–30% sucrose gradient containing (○— ○) 0.7 M NaCl or (●— ●) 4 M urea, or (×— ×) no addition. Centrifugation for 10 h at 22 000 rpm at 2 °C in a SW 27 Beckman rotor. (Trichloroacetic acid precipitable material of 100 μl samples)

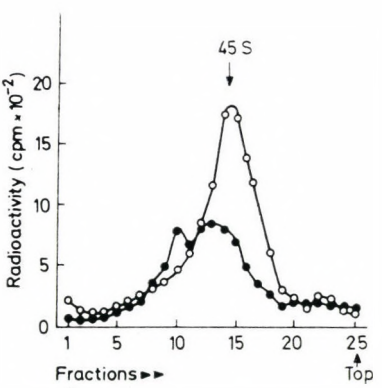

Fig. 10. Effect of 0.1 μg/ml RNAase A treatment on the sedimentation of 45*S* hnRNP complexes in a 15–30% sucrose gradient. Centrifugation for 3.5 h at 40 000 rpm at 2 °C in a SW 65 Beckman rotor. Trichloroacetic acid precipitable radioactivity of the (○— ○) control 45*S* and (●— ●) RNAase treated material. (0.1 μg/ml RNase A, at 0 °C for 10 min before resedimentation. 50 μl and 100 μl samples, respectively

Discussion

The procedure of isolation of cell nuclei from cultured *Drosophila melanogaster* cells described earlier (Szabó et al., 1981) appears to be the most reliable method for extraction and characterization of hnRNP particles. It is not easy to prepare large hnRNP particles from *Drosophila* cells. These contain a high level of endogenous RNase, and it is therefore essential to use a large amount of RNAase inhibitor. There are very long RNA stretches between monomer RNP particles (Beyer et al., 1980, 1981), giving easy access for endogenous RNAases to degrade these RNA chains.

In the present work the hnRNP complex from cultured *Drosophila melanogaster* cells was characterized biochemically and morphologically. According to our biochemical observations the hnRNP has a polysome-like structure. The subunit of the polyparticles proved to be 45*S* with a slight degree of heterogeneity. A wide profile curve for the 45*S* monomers could be obtained after sucrose gradient resedimentation (not shown). A homogeneous peak was not observed by CsCl density gradient centrifugation of the particles either. The heterogeneity of the monomer particles in density gradients may reflect differences in the size of residual RNA left on the particles or reveal the presence of different populations of particles. The density of hnRNP complexes was found to be about 1.4 g/cm^3 indicative of an RNA to protein ratio of about 1 : 4.

Our biochemical findings were confirmed by the electron microscopic data. In the 45*S* zone monoparticles were found and revealed a rather heterogeneous population with a mean size of 24 nm. The 60*S* material contained predominantly dimers, and the 90 to 100*S* region consisted of complexes containing 2 to 5 monomer particles with distinct diameters.

The protein composition of *Drosophila melanogaster* informofer was found to differ from the mammalian one in the electrophoretic mobility and the number of main polypeptides. There were no qualitative differences in protein composition between the 45*S* particles and the heavier complexes.

It was earlier described that the nuclear 30*S* hnRNP particles dissociate to RNA and proteins at relatively low salt concentrations (Samarina et al., 1967; Molnár, 1973; Gallinaro-Matringe et al., 1975). In the present experiments the dissociation of *Drosophila* hnRNP was observed as a result of increased NaCl concentration in the sucrose gradient. At a salt concentration of 0.4 M there was a shift of radioactivity to the lighter region of the sucrose gradient (not shown) and at 0.7 M almost complete dissociation of the complexes took place. In our experiments the hnRNP particles from *Drosophila* cells revealed a pronounced ribonuclease sensitivity similar to that reported for mammalian particles (Molnár, Komáromy, 1974; Stevenin et al., 1977).

The sedimentation coefficient of RNA from the 45*S* particles was about 7–14*S*. The heavier hnRNP complexes contained RNA ranging in size up to 28*S*. Neither labelling of the cells for a long (10–12 h) or short time, nor the absorbance profile at 260 nm in the sucrose gradients pointed to a contamination by rRNA of

the hnRNP RNA. There were marked differences in base composition among the RNAs from the heavier zones and especially the RNA from the 45*S* hnRNP complexes. RNA from the 45*S* particles contained mainly A and U (A + U/ /G + C = 1.75). The base composition of the RNA isolated from the polyparticles had much less A + U. These results may reveal a sequence-specific localization of protein particles along the hnRNA chain. Such a nonrandom, sequence-specific organization of nascent hnRNP was earlier assumed on the basis of electron microscopic observations using the chromatin spreading method (Peyer et al., 1980, 1981).

The results of our biochemical studies and experiments on the morphology of the hnRNP complexes confirm the above suggestion about nonrandom monomeric organization of Drosophila hnRNP complexes.

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Vanadate Inhibition of Na^+K^+. ATPase and K^+-dependent p-Nitrophenylphosphatase: a Kinetic Analysis

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Vanadate in redox state +5 inhibited the Na^+K^+-activated ATPase as well as the potassium-stimulated *p*-nitrophenylphosphatase (p-NPPase) activities of plasma membrane fragments prepared from rat brain. Vanadate exhibited a mixed type inhibition on the Na^+K^+-ATP-ase activity. The same type of inhibition was observed when the *p*-NPPase activity of the enzyme preparation was measured either in the presence of 20 mM K^+ or with 5 mM Na^+ + 1 mM K^+. When the reaction mixture contained 50 μM ATP, 5 mM Na^+ and 1 mM K^+, inhibition of *p*-NPP hydrolised by vanadate displayed a noncompetitive character. Higher noradrenalin concentration was required for counteracting the inhibition of *p*-NPPase by vanadate in the presence of ATP than in its absence.

Introduction

It is generally accepted that the K^+-dependent *p*-NPPase activity of Na^+K^+-ATPase preparations can serve as a model reaction for the K^+-dependent dephosphorylation of Na^+K^+-ATPase (EC. 3,6,1,3 ATP-phosphorylase).

The *p*-NPPase of Na^+K^+-ATPase preparations can be activated by K^+ and inhibited by Na^+ or ouabain. The two substrates, ATP and *p*-NPP show competition for the enzyme (Skou, 1974; Bader, Sen, 1966). However, under certain experimental conditions, when the total concentration of the monovalent cations used is lower than 10 mM, ATP added at micromolar concentration activates the cleavage of *p*-NPP (Yoshida et al., 1969; Somogyi, 1971; Skou, 1974). The mechanism underlying this effect of ATP has not been revealed yet.

Previous studies had shown that commercial ATP products prepared from muscle contained vanadate as a contaminant (Cantley, Josephson, 1977; Cantley et al., 1977; Ádám-Vizi et al., 1980). The presence of vanadate has been demonstrated in many tissues beside muscle, including brain (Post et al., 1979; Ádám-Vizi et al., 1980). Vanadate inhibits Na^+K^+-ATPase and K^+-dependent *p*-NPPase

Abbrevations: ATP, adenosine 5'-phosphate; ATPase, ATP-phosphohydrolase; p-NPP, p-nitrophenylphosphate

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at a very low concentration, but does not affect the Na^+-dependent phosphorylation of the enzyme (Beaugé, Glynn, 1978; Cantley, Philip, 1979). The inhibition caused by vanadate can be reduced by increasing the ATP concentration in most cases (Wu, Phillis, 1979; Ádám-Vizi et al., 1981). Therefore it seemed worth-while to investigate if the kinetics of the vanadate inhibition of *p*-NPPase would be influenced by the presence of ATP. If *p*-NPPase activity is stimulated via the formation of a phosphorylated enzyme intermediate, which cannot be inhibited by vanadate, this fact should also be reflected in an altered kinetic pattern.

Since vanadate inhibition of both Na^+K^+-ATPase and *p*-NPPase can be counteracted by noradrenalin (Wu, Phillis, 1979; Ádám-Vizi et al., 1980), the effect of noradrenalin on the vanadate inhibition of *p*-NPPase was also tested.

Materials and methods

Na^+K^+-ATPase free from Mg^{2+}-ATPase, was prepared from rat brain as described previously (Somogyi, Vincze, 1961; Somogyi, 1964). ATPase activity was measured according to Somogyi (1964).

p-NPPase activity was determined in 1 ml test volume containing 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 5 mM p-NPP (Tris-salt, Na^+-free) and different concentrations of monovalent cations as indicated in the figures.

After incubation at 37 °C for 10 min, NaOH was added at a final concentration of 0.1 M and the amount of liberated *p*-NP was measured spectrophotometrically at 405 nm. As NaOH causes oxidation of noradrenaline to noradrenochrome, it interfered with the determination of *p*-NP. Therefore, to the reaction mixtures containing 1–10 mM noradrenalin, 10 mM ascorbate was added together with NaOH to reduce the noradrenochrome formed to a leuco derivative. Under our experimental conditions the enzyme activities showed linearity over 30 minutes.

Protein content of enzyme preparation was determined by the Biuret method as described by Gornall et al. (1949), using bovine serum albumin as standard.

L-noradrenalin was obtained from BDH Chemicals Ltd., Poole, L-ascorbic acid was the product of Merck (Darmstadt), serum albumin was that of Calbiochem (Luzern), vanadate-free ATP was purchased from Sigma (St. Louis) and all other reagents from Reanal, Budapest.

Results

Inhibition of the Na^+K^+-ATPase activity by vanadate

Vanadate inhibited the Na^+K^+-ATPase activity in membrane fragments of rat brain causing a progressive decrease in affinity of ATP to the enzyme. The K_m for ATP was 0.3 mM in the absence of vanadate and this value was increased by increasing vanadate concentrations (Fig. 1). Half maximal inhibition of the Na^+K^+-ATPase activity was measured at a vanadate concentration of approx.

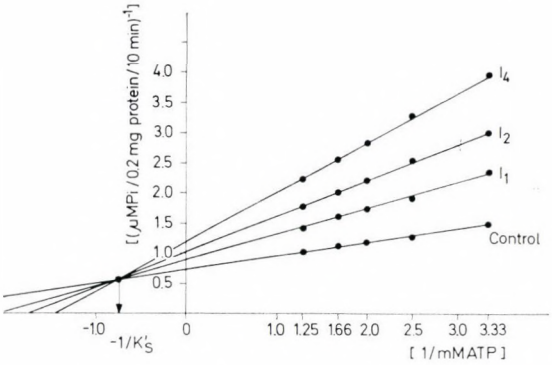

Fig. 1. Lineweaver-Burk plot of the vanadate inhibition: The medium contained 50 mM Tris. HCl, pH 7.4, 5 mM $MgCl_2$; 100 mM NaCl, 20 mM KCl and 0.1 mg/ml of plasmamembrane fraction. Substrate concentrations: 0.3, 0.4, 0.5, 0.6 and 0.8 mM ATP. Inhibitor concentrations: I_1= 0.1; I_2 = 0.2 and I_4 = 0.4 uM $NaVO_3$. Values of linear regression: r_c = 0.9924; r_{I_1} = 0.9965; r_{I_2} = 0.9995; r_{I_4} = 0.9988; K_m = 0.3 mM ATP

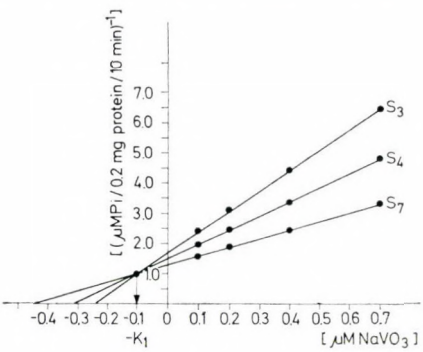

Fig. 2. Dixon plot of the vanadate inhibition: The medium contained 50 mM Tris. HCl, pH 7.4, 5 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl and 0.1 mg/ml of plasma membrane fraction. Substrate concentration: S_3 = 0.3; S_4 = 0.4 and S_7 = 0.7 mM ATP. Inhibitor concentrations: 0.1, 0.2, 0.4 and 0.7 μM $NaVO_3$. Values of linear regression: r_{S_7} = 0.9995; r_{S_4} = 0.9985; r_{S_3} = 0.9974; K_I = 0.1 μM $NaVO_3$

0.1 μM (Fig. 2). An analysis of the kinetic parameters showed that the inhibition of Na^+K^+-ATPase by vanadate was of a mixed type as shown in Lineweaver-Burk plots (Fig. 1).

The effect of vanadate on the K^+-dependent p-NPPase activity of a Na^+K^+-ATPase preparation

The Na^+K^+-ATPase preparation used in our experiments contained K^+-stimulated *p*-NPPase. The specific activity of the latter was 5% that of Na^+K^+-ATPase. Maximal *p*-NPPase activity was measured in the presence of 20 mM K^+

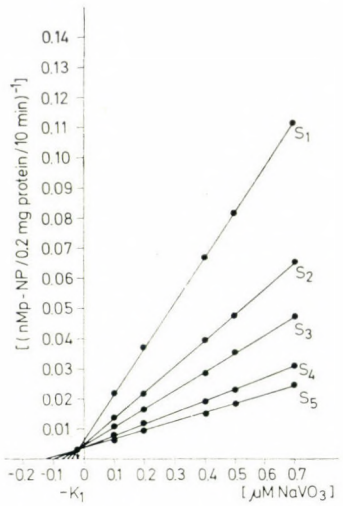

Fig. 3. Dixon plot of the vanadate inhibition: The medium contained 50 mM Tris. HCl, pH 7.4, 5 mM $MgCl_2$, and 20 mM KCl and 0.1 mg/ml of plasma membrane fraction. $S_1 = 1$, $S_2 = 2$, $S_3 = 3$, $S_4 = 4$ and $S_5 = 5$ mM *p*-NPP. Inhibitor concentration: 01, 0.2, 0.4, 0.5 and 0.7 μM $NaVO_3$. Values of linear regression: $r_{S_1} = 0.9999$; $r_{S_2} = 0.9998$; $r_{S_3} = 0.9978$; $r_{S_4} = 0.9999$; $r_{S_5} = 0.9999$; $K_I = 0.02$ μM $NaVO_3$

(Somogyi, 1971). Vanadate ions inhibited *p*-NPPase activity. Fifty per cent inhibition was measured in the presence of 0.02 μM vanadate (Fig. 3). Thus the vanadate sensitivity of *p*-NPP cleavage was about five times higher then that of ATP hydrolysis. This was also indicated by a comparison of the apparent K_m values. The K_m for *p*-NPP in the absence of vanadate was approximately 2 mM which increased to 10.4 mM in the presence of 0.4 μM vanadate. Consequently, the affinity of vanadate to appropriate binding sites is considerably higher than that of *p*-NPP. The vanadate inhibition of *p*-NPPase also displayed kinetics of the mixed type (Fig. 4).

Effect of vanadate on the p-NPPase activity in the presence of ATP and low concentration of monovalent cations

ATP in the micromolar range enhanced the *p*-NPPase activity both in the absence and in the presence of very low concentrations of Na^+ or K^+; maximal activity was measured with 5 mM Na^+ + 1 mM K^+ + 50 μM ATP added simultaneously (Somogyi, 1971).

Table 1 demonstrates that in the presence of ATP at the optimal concentration, the rate of *p*-NPPase activity is enhanced by 300 % as compared to the control. In the presence of 5 mM Na^+ + 1 mM K^+ the K_m value was 1.25 mM for *p*-NPP that increased to 11.1 mM in the presence of 50 μM ATP. However, a kinetic analysis of the vanadate inhibition showed substantial differences when the

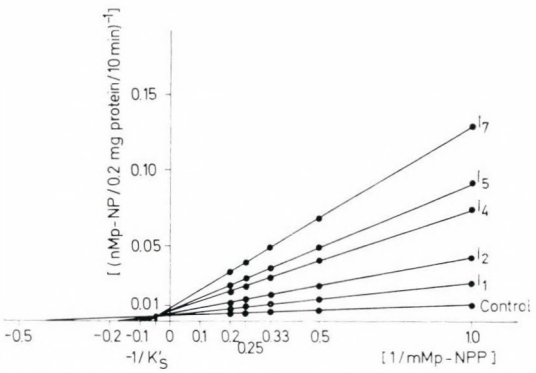

Fig. 4. Lineweaver-Burk plot of the vanadate inhibition: The medium contained 50 mM Tris. HCl, pH 7.4, 5 mM $MgCl_2$, 20 mM KCl and 0.1 mg/ml of plasma membrane fraction. Substrate concentrations: 1, 2, 3, 4 and 5 mM p-NPP. Inhibitor concentrations: $I_1 = 0.1$, $I_2 = 0.2$, $I_4 = 0.4$, $I_5 = 0.5$ and $I_7 = 0.7$ μM $NaVO_3$. Values of linear regression: $r_c = 0.9737$; $r_{I_1} = 0.9975$; $r_{I_2} = 0.9985$; $r_{I_4} = 0.9978$; $r_{I_5} = 0.9999$; $r_{I_7} = 0.9986$; $K'_S = 20$ mM *p*-NPP

Table 1

Dependence of p-NPP cleavage on the concentration of ATP

Reaction mixture: 50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 5 mM NaCl, 1 mM KCl, 5 mM p-NPP, 0.1 mg/ml of plasma membrane fraction and ATP as indicated. Each value represents the mean of two experiments with 2 parallels

Sample	Ions present in the media			
	Mg^{2+}, Na^+, K^+	Mg^{2+}	Mg^{2+}, Na^+	Mg^{2+}, K^+
	nM p-NP/0.2 mg protein/10 min			
Control	61.2	12.1	17.6	52.1
25 μM ATP	137.5	58.8	80.0	81.5
50 μM ATP	194.3	64.2	93.1	68.2
100 μM ATP	139.2	43.3	79.5	62.1
1000 μM ATP	61.5	16.8	28.0	13.9

p-NPPase activity was measured either with or without 50 μM ATP. Without ATP, vanadate caused again a mixed type inhibition with $K'_S = 7.1$ mM for *p*-NPP and $K_I = 0.085$ μM for vanadate. The affinity of *p*-NPPase to *p*-NPP decreased in the presence of vanadate: the K_m value was increased 4 times by 0.5 μM vanadate in comparison with the control (Figs 5 and 6). In the presence of 50 μM ATP, the vanadate inhibition of the *p*-NPPase showed a noncompetitive character, when analyzed by either the Lineweaver-Burk or the Dixon plot, with $K'_S = 11.1$ mM for *p*-NPP and $K_I = 0.086$ μM for vanadate. However, it should be pointed out that the decreased affinity of *p*-NPPase to *p*-NPP in the presence of

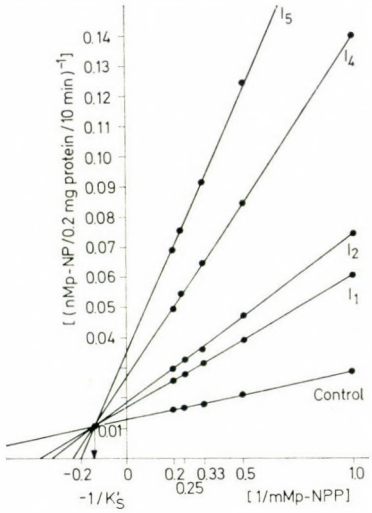

Fig. 5. Lineweaver-Burk plot of the vanadate inhibition: The medium contained 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 5 mM NaCl, 1 mM KCl and 0.1 mg/ml of plasma membrane fraction. Substrate concentrations: 1, 2, 3, 4 and 5 mM p-NPP. Inhibitor concentrations: $I_1 = 0.1$, $I_2 = 0.2$, $I_4 = 0.4$ and $I_5 = 0.5$ μM $NaVO_3$. Values of linear regression: $r_c = 0.9958$; $r_{I_1} = 0.9997$; $r_{I_2} = 0.9854$; $r_{I_4} = 0.9959$; $r_{I_5} = 0.9999$; $K_s' = 7.1$ mM *p*-NPP

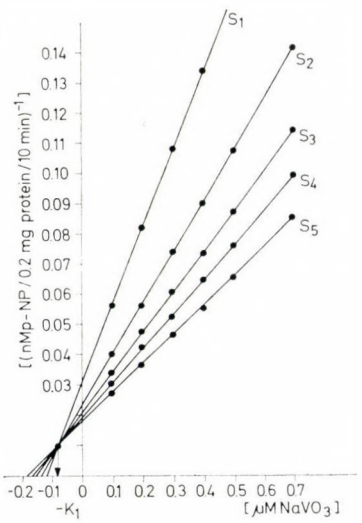

Fig. 6. Dixon plot of the vanadate inhibition: The medium contained: 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 5 mM NaCl, 1 mM KCl and 0.1 mg/ml of plasma membrane fraction. Substrate concentrations: $S_1 = 1$, $S_2 = 2$, $S_3 = 3$, $S_4 = 4$, and $S_5 = 5$ *p*-NPP. Inhibitor concentrations: 0.1, 0.2, 0.4, 0.5 and 0.7 μM $NaVO_3$. Values of linear regression: $r_{S_1} = 0.9972$, $r_{S_2} = 0.9999$, $r_{S_3} = 0.9999$, $r_{S_4} = 0.9999$ and $r_{S_5} = 0.9995$. $K_I = 0.085$ μM $NaVO_3$

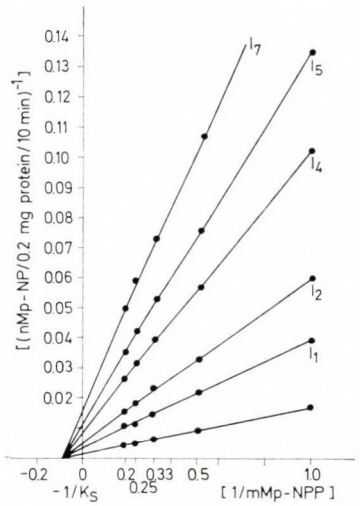

Fig. 7. Lineweaver-Burk plot of the vanadate inhibition: The medium contained 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 5 mM NaCl, 1 mM KCl, 50 μM ATP and 0.1 mg/ml of plasma membrane fraction. Substrate concentrations: 1, 2, 3, 4 and 5 mM p-NPP. Inhibitor concentrations: $I_1 = 0.1$, $I_2 = 0.2$, $I_4 = 0.4$, $I_5 = 0.5$ and $I_7 = 0.7$ μM $NaVO_3$. Values of linear regression: $r_c = 0.9980$; $r_{I_1} = 0.9980$; $r_{I_2} = 0.9994$; $r_{I_4} = 0.9993$; $r_{I_5} = 0.9990$; $r_{I_7} = 0.9995$; $K_s' = 11.1$ mM *p*-NPP

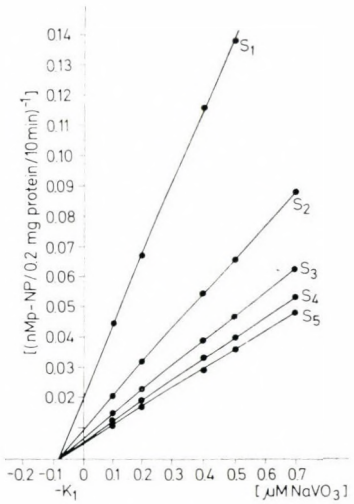

Fig. 8. Dixon plot of the vanadate inhibition: The medium contained 50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$ 5 mM NaCl, 1 mM KCl, 50 μM ATP and 0.1 mg/ml of plasmamembrane fraction. Substrate concentrations: $S_1 = 1$, $S_2 = 2$, $S_3 = 3$, $S_4 = 4$ and $S_5 = 5$ mM *p*-NPP. Inhibitor concentrations: 0.1, 0.2, 0.4, 0.5 and 0.7 μM $NaVO_3$. Values of linear regression: $r_{S_1} = 0.9995$; $r_{S_2} = 0.9998$; $r_{S_3} = 0.9990$; $r_{S_4} = 0{,}9995$; $r_{S_5} = 0{,}9992$; $K_I = 0.0863$ μM $NaVO_3$

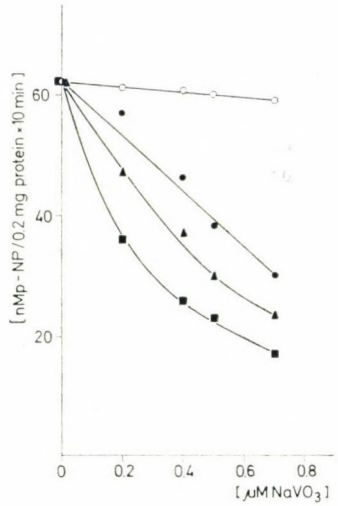

Fig. 9. Inhibitory effect of $NaVO_3$ on *p*-NPPase in the presence of noradrenalin at different concentrations. Reaction mixture: 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 5 mM NaCl, 1 mM KCl and 0.1 mg/ml of plasma membrane fraction. ■— ■, none; ○— ○, 10^{-3} M noradrenalin; ●— ●, 10^{-4} M noradrenalin; ▲— ▲, 10^{-5} M noradrenalin

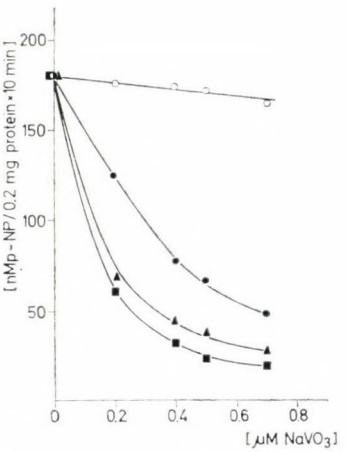

Fig. 10. Inhibitory effect of $NaVO_3$ on *p*-NPPase in the presence of 50 μM ATP and noradrenalin at different concentrations. Reaction mixture: 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 5 mM NaCl, 1 mM KCl, and 0.1 mg/ml of plasma membrane fraction. ■— ■, none; ○— ○, 10^{-3} M noradrenalin; ●— ● 10^{-4} M noradrenalin; ▲— ▲, 10^{-5} M noradrenalin

50 μM ATP could not be further reduced by vanadate because the apparent K_m value was the same in the presence and absence of vanadate (Figs 7 and 8).

Effect of noradrenalin on the inhibition of p-NPPase by vanadate

When p-NPPase activity was assayed in the presence of 5 mM Na^+ + 1 mM K^+ with or without 50 μM ATP the extent of hydrolysis of *p*-NPP was not affected by noradrenalin at concentrations ranging from 10 to 1000 μM (Figs 9 and 10). When the reaction mixture contained vanadate, its inhibitory effect could be diminished or even abolished by increasing the concentration of noradrenalin. In the presence of 1000 μM noradrenalin, 0.7 μM vanadate did not inhibit *p*-NPPase either in the presence or in the absence of ATP.

However, when noradrenalin was applied at lower concentrations, the extent of counteracting effect of noradrenalin depended on the presence of ATP: the inhibitory effect of vanadate was more pronounced in the presence of ATP than in its absence.

Discussion

Previous experiments revealed that vanadium in its 5^+ oxidation state inhibited in vitro the Na^+K^+-ATPase, as well as the K^+-stimulated *p*-NPPase activity (Cantley et al., 1977; 1978a,b; Beaugé, Glynn, 1978; Beaugé, DiPolo, 1979a,b; Karlish et al., 1979; Wu, Phillis, 1979).

Kinetic analyses of the vanadate effect in different laboratories led to conflicting results concerning the type of inhibition. Thus, for example Wu and Phillis (1979) described the vanadate inhibition of Na^+K^+-ATPase as uncompetitive, while that of *p*-NPPase as competitive in rat brain homogenate. In contrast, in the synaptosome fraction of rat brain, competitive inhibition of Na^+K^+-ATPase by vanadate was demonstrated (Ádám-Vizi et al., 1981). In the present experiment we used a plasma membrane fraction of rat brain in which the ATPase activity could only be measured in the simultaneous presence of Mg^{2+} + Na^+ + + K^+, and which did not contain Mg^{2+} – ATPase (Somogyi, 1964). The vanadate inhibition of Na^+K^+-ATPase in this preparation was of the mixed type, similar to that of *p*-NPPase, in both cases under optimal conditions e.g. in the presence of 20 mM K^+, or 5 mM Na^+ and 1 mM K^+. After addition of 50 μM ATP to the reaction mixture containing 5 mM Na^+ and 1 mM K^+, the type of vanadate inhibition of *p*-NPPase became noncompetitive.

The exact mechanism of the vanadate inhibition of the Na^+ + K^+-ATPase and that of its partial reaction are not known in detail. Kinetic measurements demonstrated the existence of two binding sites of Na^+ + K^+-ATPase for vanadate. Under optimal conditions (high concentrations of Mg^{2+} and K^+ and omission of Na^+ or ATP) one binding site exhibits high affinity, the other one low affinity for vanadate. When different ligands (Mg^{2+}, Na^+, K^+, P_i and ATP or pNPP or both) are present in the incubation medium, the binding and therefore the inhibitory action of vanadate will be modified. This modifying effect depends to

a large extent not only on the presence of other ligands but also on their concentrations applied. In various plasma membrane preparations containing $Na^+ + K^+$-ATPase activity, the proportion if in-side-out to right-side-out vesicles is quite different. However, vanadate binds only to the cytoplasmic side of the enzyme (Cantley et al., 1978a) and cannot affect the pohosphorylation step of $Na^+ + K^+$-ATPase (Beaugé, 1979). On the other hand, stimulation of *p*NPP hydrolysis by low concentration of ATP seems to be somehow related to enzyme phosphorylation. Summing up, all these factors can determine not only the extent of inhibition of the enzyme by vanadate but probably also influence the type of vanadate inhibition by changing the affinity of vanadate to its binding sites. Because these not exactly determined factors are different in the various experiments published, it is expected that they could account for the very divergent results concerning the type of enzyme inhibition by vanadate.

Comparing our results with those of Cantley et al. (1979) a model can be put forward to interpret the described effects of vanadate. When the system contained only one of the substrates the inhibition caused by vanadate was of the mixed type. This can be considered as the result of a competitive and a noncompetitive inhibition. Cantley et al. (1979) suggest that the $Na^+ + K^+$-ATPase possesses two common binding sites for ATP and vanadate. It can be supposed that under our experimental conditions the affinities of the two binding sites for vanadate are nearly the same, while the substrates are bound with different affinities. Hydrolysis takes place primarily at the noncompetitive binding site. If the vanadate inhibition is complete, both sites are inhibited by the anion. Thus both noncompetitive and competitive types of inhibition take place, the sum of which is displayed as a mixed type inhibition. For the interpretation of the stimulatory effect of ATP, one may assume that either ATP itself or the phosphoenzyme generated by its hydrolysis, alters the conformation of the ATPase in such a way that the inhibition by vanadate at the competitive binding site is abolished while the noncompetitive site can be still inhibited, resulting in a noncompetitive inhibition.

It is well known that noradrenalin can reduce vanadate (Ádám-Vizi et al., 1981). Examining the effect of noradrenalin on the vanadate inhibition of *p*-NPPase we observed that in the presence of ATP a higher concentration of noradrenalin was necessary to abolish inhibition than in the absence of ATP. This phenomenon can be explained by assuming that, because of the vanadate binding to the competitive binding site, that is idle due to a conformational change, binding of the noradrenalin-vanadate complex is restricted, whereas in the absence of ATP, vanadate becomes more accessible to noradrenalin.

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Assembly and Reliability of an X-ray Microanalyser System with a Possibility for Independent Mass Measurement

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A new X-ray microanalyser assembly is described, the main parts of which are a JEOL 100 B transmission electron microscope, a JEOL ASID I scanning attachment, an EDAX 183 B semiconductor detector and a KFKI ICA 70 4k multichannel analyser. By using this equipment, qualitative analysis, equivalent to the original EDAX version, can be performed. Furthermore it is possible to visualize simultaneously the spatial distribution of two or more elements in the sample. The mass of the excited volume of the sample can be determined, independently of the X-ray measurements, by using the electron detector of the scanning attachment. The reproducibility of such measurements is demonstrated and a calibration curve is given. By recording the excited mass of the sample during the X-ray analysis, the damage caused by the exciting electron beam was also examined.

Introduction

Although the X-ray microanalytical technique has a comparatively short history (Castaing, Guinier, 1949), it plays an important and continuously increasing role in the examination of both biological and inorganic materials. For a general review of the present state of X-ray microanalysis, and its relation to other micro-analytical methods, the paper of Newbury (1979) can be consulted. In an other recent review, Chandler (1979) has critically reexamined the existing problems of biological microanalysis. Some biological applications can be found in the paper of Tóth (1980).

In the present paper, a new instrument assembly is proposed in which the pulses of the X-ray detector are fed to an universal multichannel analyser (MCA) – so, besides the possibilities offered by the original instruments (e.g. ORTEC, EDAX), the versatility of the MCA can be used to solve additional problems.

The solution of some special problems requires the identification of compounds rather than that of single atoms. In such cases the distribution of all elements or rather the relative distribution of one element in comparison to the others should

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be determined. A possible way of determining the spatial distribution of elements with our instrument, and the simultaneous mapping of two or more elements is described. These are qualitative examinations only.

The quantitative analysis of thin samples imposes certain difficulties. One of the most important problems is the inhomogeneity of the section thickness measured on the scale of the beam diameter. Further problems arise from the damage caused by the electron beam. As the contamination effect is caused mainly by the environment of the sample and not its intrinsic properties, in the case of thin samples the relative mass change during analysis (increase in mass in comparison to the initial mass of the irradiated volume) may be large. To overcome these difficulties, a method is described which is suitable to correct the errors arising from the local inhomogeneities of the sample, and to trace the mass change during analysis.

Materials and methods

The basic parts of the assembly are: a JEOL 100 B transmission electron microscope with a JEOL ASID I scanning unit attached to it, an EDAX 183 Si(Li) detector with a preamplifier/amplifier and a KFKI ICA 70 4k multichannel analyser (Fig. 1).

The X-ray detector

The 183 B type detector is a semiconductor detector equipped with a preamplifier and amplifier unit. There are two signals at the output of the amplifier: (*i*) the signal of the fast channel which, for simplicity, is called fast discriminator (FD) signal, and (*ii*) the signal of the slow or measuring channel (M pulses). The amplitude (4.5 V) and the duration (500 ns) of the FD pulses are constant; their function is to indicate the outcoming slow pulses. The signals of the measuring channel are Gaussian pulses with fixed full width at half of maximum (FWHM) and amplitudes proportional to the energy of the X-ray photons to be measured.

The principles and problems of measurements with Si(Li) detectors are discussed in the papers of Statham (1980, 1981).

The multichannel analyser

The MCA used is able to analyse amplitudes and to store spectra with a resolution of 4k, 2k and 1k. Even in the worst case, the contribution of the MCA to the total resolution of the measuring chain is negligible. During amplitude analysis a start signal opens the gate at the input of the MCA and the amplitude maximum appearing at the input during 5 μs counting from this moment will be stored. The start signal may be either an external pulse or itself the pulse to be measured. At the input there is an upper/lower variable discriminator too – measurement will occur only when the amplitude of the signal is between the discriminator levels. For every pulse measured, a TTL output signal is available.

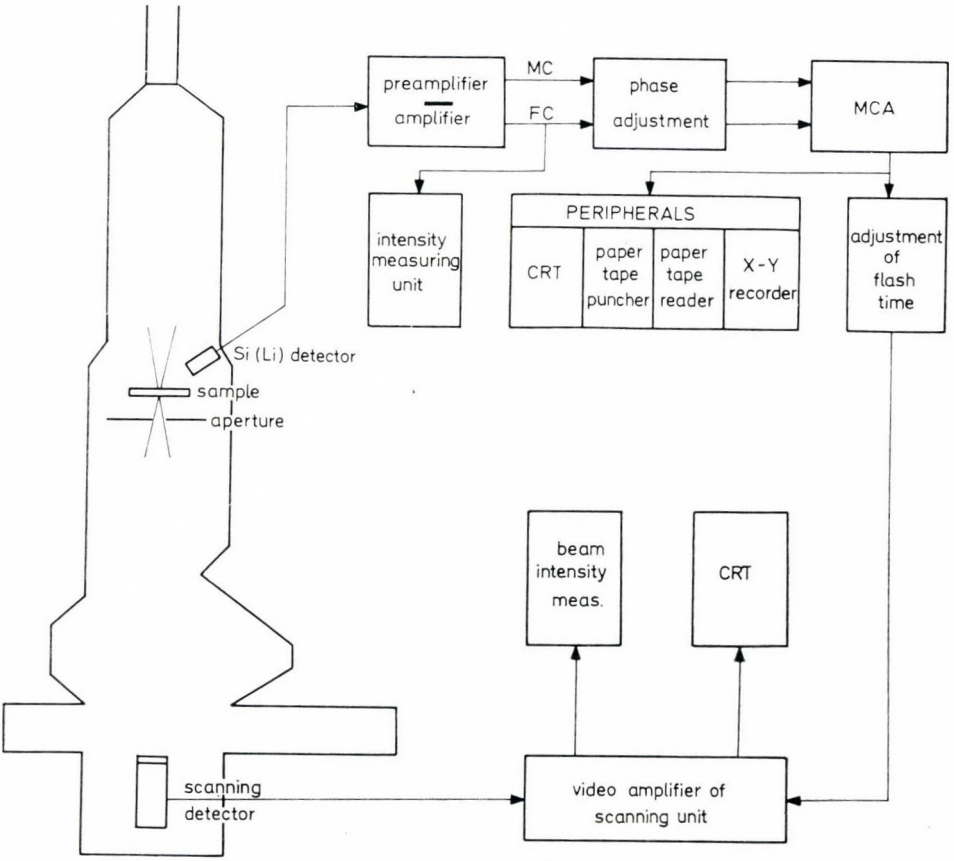

Fig. 1. Block diagram of the microanalyser assembly. The signals of the measuring channel (MC) and the FD pulses of the fast channel (FC) after phase adjustment are fed to the MCA for analysis. By using the existing peripherals of the MCA "off line" computer analysis can be performed. About the meaning of the other boxes see the text

The coupling unit

The main pulses of the detector amplifier are too broad (60 μs) compared to the opening time of the input gate of the MCA (5 μs), so the internal sampling possibility of the analyser is not appropriate to start the measurements. As the quantity to be measured by the MCA is the amplitude of an M pulse, external signals starting the A/D conversion at its maximum should be used. The time delay between an FD signal and the corresponding main pulse is constant, therefore start signals can be easily made from the FD pulses with the aid of two monostable multivibrators (Fig. 2).

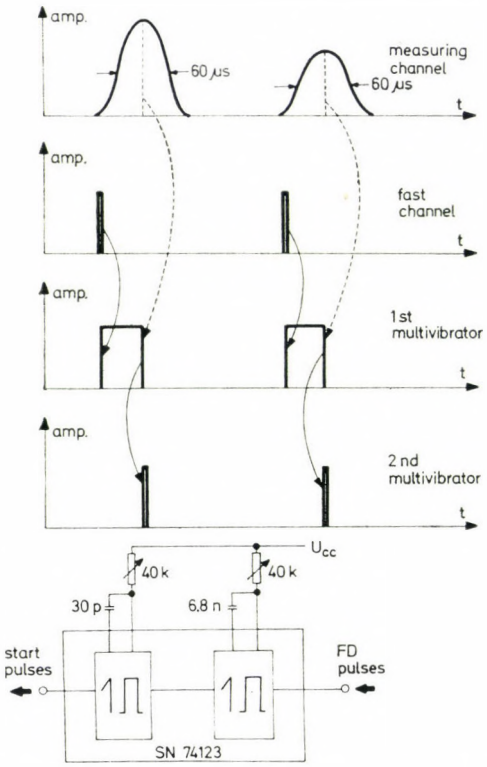

Fig. 2. Phase diagram of the signals of the coupling unit. The first multivibrator is triggered by the FD signals and its duration time is chosen in such a way that the high-to-low transition coincides with the amplitude maximum of the main pulse. The second multivibrator driven with this edge gives a start signal for the MCA. At the bottom of the figure the schematic diagram of this unit is shown

Measurement of section thickness

Sections were made from synthetic resin (Araldit), which is similar to the biological sections, as far as the interaction with the illuminating beam is concerned. The sections (collected on Formvar coated copper grids) were exposed to well collimated (100–200 nm) beam for 10–50 s in scanning transmission mode, till the contamination cones appeared on both sides of the sections. The appearance of the contamination cones was verified by visual examination of the sample in conventional transmission mode, when they could be recognized as dark spots. The cones on the upper and lower side of the specimen can be distinguished from each other if the direction of the observation is different from the direction of the probing (Fig. 3). Consequently, after tilting the specimen to a known angle, the speci-

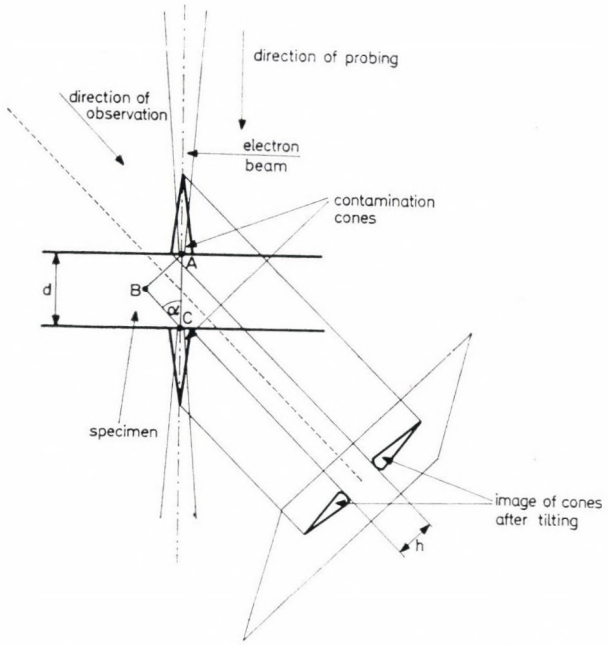

Fig. 3. Determination of the specimen thickness. The thickness can be determined by tilted observation of the contamination cones obtained during excitation the specimen with a well collimated electron beam. The thickness can be readily obtained after substituting the distance between the projected image of the cones (h) and the tilting angle (α) into Eq. 1. which was derived from the ABC right-angled triangle

men thickness can be calculated according the following formula:

$$d = \frac{h}{\sin \alpha} \tag{1}$$

where α is the tilting angle, h is the distance between the bases of the contamination cones on the image and d is the specimen thickness. The h distance was determined by evaluation of micrographs recorded during conventional microscopy (Goodhew, Chescoe, 1980).

Results and Discussion

Qualitative spot analysis

As all parts of the detector-amplifier-MCA chain are linear, the channel number in the obtained spectrum is linearly proportional to the X-ray energy. This means that the unknown characteristic lines can be identified by using the two point calibration method. As the perturbing effect of the environment of the instrument

(e.g. temperature change) may introduce considerable peak shift (1 K change leads to a shift of 5 eV at 10 keV) (Statham, 1981), it is necessary to calibrate the energy axis at least once in a series of measurements.

Dealing with thin biological samples, the X-ray intensity reaching the detector is usually not higher than 100–500 cps so the deformation of spectra caused by the pulse pile-up is not greater than 1–2%. Among other harmful effects (e.g. contamination) this is negligible.

As compared to the original version in this new application of the FD pulses the phase stability between the FD signals and the main pulses is much more important. Phase instability may introduce peak broadening or a decrease in resolution in the final spectrum, so we checked the resolution of this assembly. To do this, for simplicity, the actual energy calibration spectrum was used in each case and the FWHM value at the energy of Mn_{K_α} was transformed to the energy of Al and Cu (the calibration standards) by means of the following equation:

$$R_x = \sqrt{R_{Mn}^2 + k(E_x - E_{Mn})} \tag{2}$$

which describes the total resolution of the assembly as a function of the photon energy (R_x, R_{Mn} are the resolutions at E_x and E_{Mn} respectively; $k = cFV$, where F is the Fano factor, V is the mean ionisation potential of the X-ray detector and c converts the standard deviation of the Gaussian distribution into FWHM value). This relationship contains the constant noise contribution to the total resolution (included in the R_{Mn}^2 term) and the energy dependent detector resolution factor (second term). As the k coefficient is characteristic of the detecting unit only and not of the signal processing part of the assembly, $k = 2.375$ value was used, which was obtained with the original instrumentation (Jánossy et al., 1979). The original resolution at Mn_{K_α} obtained by the EDAX 707 B analyser was 165 eV, the calculated values at Al_{K_α} and Cu_{K_α} were 123 eV and 182 eV, respectively. The characteristic values of our instrument are 122 eV at Al_{K_α} and 184 eV at Cu_{K_α}. The comparison of the corresponding values clearly shows that the use of FD signals as start pulses for amplitude measurement does not cause decrease of resolution.

Further advantage of this assembly is the greater channel number of the MCA which is useful in Gaussian analysis of the spectra and in deconvolution of overlapping peaks.

With this assembly successful measurements have already been made on the cerebral cortex of cats (Joó et al., 1981), and on human kidney biopsies (Mágori et al., 1983).

Scanning analysis

Setting the discriminator levels of the MCA around the amplitude of a given characteristic line according to the FWHM, A/D conversion will occur (apart from the Bremsstrahlung, the energy of which is in the selected range) only when a chosen characteristic radiation reaches the detector. Consequently, an output pulse from the MCA will appear only when the selected element emits radiation. Driving

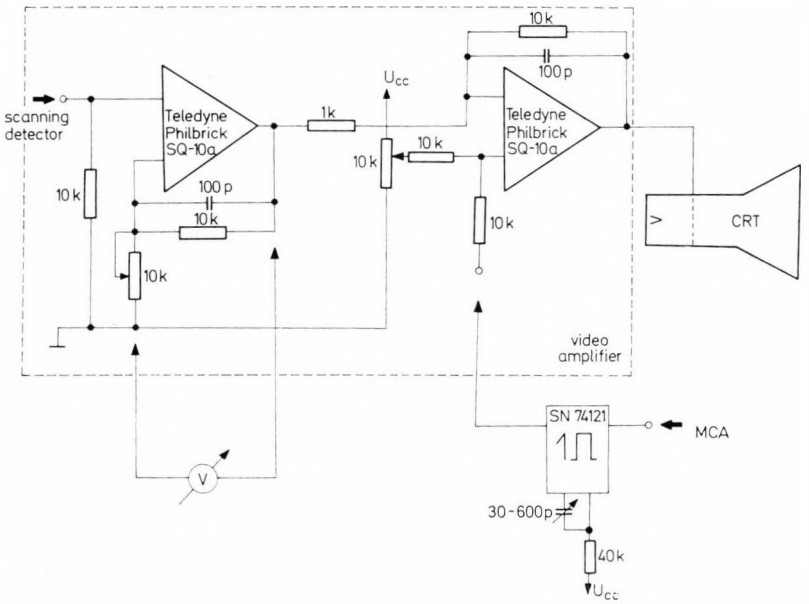

Fig. 4. Schematic diagram of the video amplifier of the scanning unit. *(i)* With a voltmeter connected to the output of the first amplifier a voltage proportional to the output of the electron detector can be measured. *(ii)* Connecting the MCA through a multivibrator to the second amplifier and driving the CRT with this signal, instead of the signal of the electron detector, strokes of different length can be produced depending on the duration time of the multivibrator and the scanning speed

the video amplifier of the scanning unit with these pulses – instead of the signal from the scanning detector – we obtain a flash on the CRT at the moment when the scanning beam excites the element of interest. As the motion of the electron beams in the microscope and on the CRT is synchronous, in such way we can demonstrate the spatial distribution of the element emitting the chosen characteristic radiation in any scanning mode of the microscope.

For simultaneous mapping of two or more elements, we should have used several detectors or a detector equipped with multichannel amplifier (Johns, Skyring, 1980). A further advantage of our instrument combination is that this can be performed by using only one detector. By inserting a monostable multivibrator with variable duration time between the video amplifier and the MCA, we can change the flash time on the CRT of the scanning unit (Fig. 4). Taking two or more scans successively with discriminator levels corresponding to the chosen elements and selecting different duration times of the monostable multivibrator for each discriminator level, by photographing the CRT in the case of each scan on the same negative, we can obtain a multielement map in which the elements are represented with strokes of different length.

Quantitative analysis

In quantitative calculations, we have to refer the characteristic X-ray intensity to the local mass of the sample. Here we propose an independent method for mass measurement which can be used beside, or instead of, the conventional calculations. If there is a possibility to measure the attenuation of the beam passing through the sample the local mass thickness can be determined according the Bouguer – Lambert – Beer law

$$I_t = I_o e^{-Sd\rho} \tag{3}$$

(I_o the incident, I_t the transmitted beam intensity, respectively; the total (elastic and inelastic) mass scattering cross section $S = \sigma N/A$, where σ is the total atomic cross section, N is the Avogadro number and A is the atomic weight. ρ denotes the density of the irradiated volume, and d is the local section thickness. ρ d is often referred as mass thickness.)

To determine the local mass thickness of the sample according to Eq. 3. we can choose from several methods which are appropriate to measure the electron beam intensity. In the scanning transmission electron microscopes the transmission electron current can be measured by using either bright field or dark field detectors (Wall, 1979), but in transmission electron microscopes with an attached scanning unit, the scanning detector is appropriate for this purpose (Halloran et al., 1978; Reimer, Hageman, 1977). In microscopes not suited for current measurements a removable electron detector can be used (Nicholson, 1981; Turner et al., 1975), but without any modification the transmitted beam intensity can be measured indirectly by densitometric evaluation of electron microscopic negatives.

In our assembly, the electron detector of the scanning attachment is used to determine the transmitted beam intensity. Using the microscope in scanning transmission mode, a voltage proportional to the electron beam intensity reaching the scanning detector can be measured by coupling a voltmeter to the output of the first step of the video amplifier of the scanning unit (Fig. 4). An output voltage which is proportional to the transmitted beam intensity ($U_t = kI_t$) can be measured when the specimen is inserted into the column. The transmitted intensity without sample is considered as incident intensity, so after retracting the specimen from the beam path the output voltage is $U_o = kI_o$. Since the k proportionality factor is the same in both case, after substituting into eg. 3. it cancels out. It is evident that the exciting beam and the electron optics of the microscope must be kept constant during the measurement.

The $Sd\rho$ value of the sample can be obtained after substitution I_t and I_o into eq. 3. To determine the mass thickness of the sample, the knowledge of the total mass scattering cross section (S) is necessary. As S is the function of the composition of the sample and mainly depends on the electron optics and the accelerating voltage, in the case of fixed electron optical parameters (including the accelerating voltage) for a given type of specimens it can be regarded as instrumental constant. Furthermore, as the function of S versus the atomic number is roughly

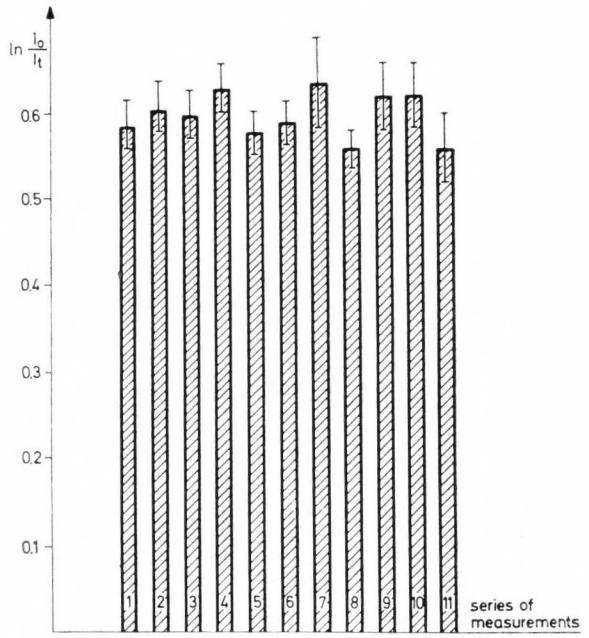

Fig. 5. Reproducibility of the mass measurements. In I_o/I_t is a quantity proportional to the mass thickness of the sample. On the horizontal axis each column represents a series of measurements which was performed with unchanged alignment of the microscope. The different lenght of columns represents the effect of realignment

constant (Burge, Smith, 1962), dealing with biological samples (the majority of the samples is the organic matrix) S can be regarded as constant, independently of the composition of the sample. Consequently, for a given set of electron optical parameters, it is enough to determine the value of S only once, by measuring a specimen of known thickness and density. Then the excited mass of the sample can be obtained by multiplication of the mass thickness obtained from eq. 3. with the exciting beam diameter.

The claim to compare results obtained at different occasions requires the stability of the electron optics or the reproducibility of the alignment of the microscope. If the reproducibility is not so good, the S and k parameters are valid for one series of measurements only. Therefore, the reproducibility of the mass measurement was rigorously checked by repeated measurement of one sample. Eleven series of measurements were done; between each series the microscope was totally realigned. Each series consisted of twelve individual measurements (Fig. 5). It can be clearly seen that the parameters are well reproducible, so we can use a single calibration curve instead of standards after each series. In other words, this means that the k proportionality factor between I and U, and the mass scattering cross section (S) do not depend on the realignment of the microscope between

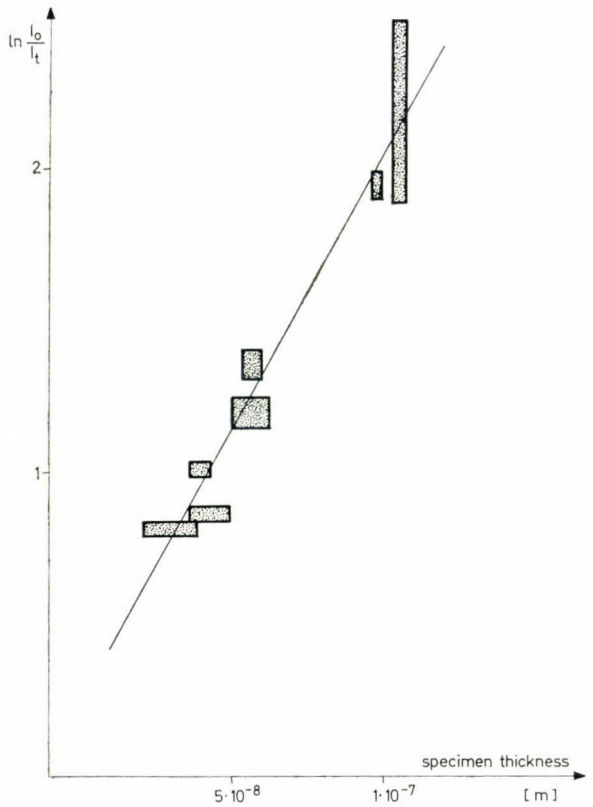

Fig. 6. Mass thickness calibration curve. In I_o/I_t (which is proportional to the mass thickness) is plotted against the section thickness. The side lenghts of rectangles represent the error of measurements. Each vertical value was averaged from 12 measurements, the horizontal values were averaged from 4 different experiments. The calibration curve was obtained with beam diameter of 100 nm, 40 μm condenser aperture and 0.78 A objective lens current. A 120 μm scanning contrast aperture was used

measurements to be compared. A calibration curve, which was obtained with the most frequently used parameters during X-ray microanalysis is shown in Fig. 6.

Every microscope has its individual electron optical geometry. Taking this into account, microscopists (probably having different experience in alignment of the microscope) would be advised to work out the calibration curve of their own microscopes rather than apply a general curve which had been obtained with a different type of microscope.

The relatively large standard deviation of mass measurements with fixed electron optics (Fig. 5) is probably due to damage of the sample during irradiation. It is clear that among the methods, based on eq. 3. techniques involving larger irradiated area will cause less damage to the sample. Therefore, the determination of the transmitted beam intensity, either by evaluating the negatives (Bahr, Zeitler

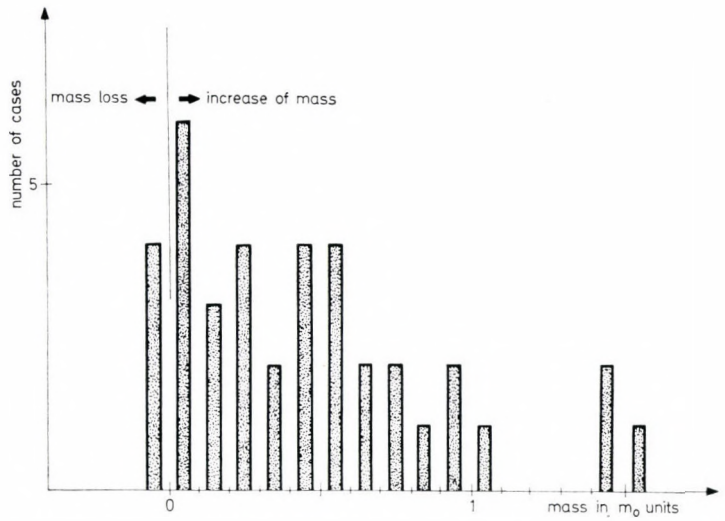

Fig. 7. Specimen damage during analysis. The initial mass of the irradiated volume was denoted by m_o, the analysing time was 100 s. It is clear that during 100 s probing, which is necessary to collect enough counts for X-ray analysis, the relative mass change may be significant

1965) or by direct measurement of the transmitted current in conventional transmission mode (Edie, Karlsson, 1972), is advantageous. (It is worth mentioning, that the accuracy of these methods is twice better (Linders et al., 1981) than that of the continuum method (Hall, 1971), i.e. the standard deviation of the continuum method is twofold greater than that of this independent mass measuring method. However, from the point of view of X-ray microanalysis, preference should be given to the mass measuring methods dealing with well focused (diameter of 10–50 nm) probes, because this strong exciting condition is exactly suitable for microanalysis. Thus, just those changes in mass can be revealed which are inherents of microanalysis under such (electron density and vacuum) conditions. Further advantages of the use of such probe are that (*i*) in such way exactly the excited mass of the sample can be determined and (*ii*) the mass measurement can be carried out during the X-ray analysis, thereby one can follow the changes from the beginning to the end of the analysis. Knowing the degree of mass change during analysis, there is a possibility to correct our results considering this effect (Marshall, 1980).

In Fig. 7 the effect of 100 s probing on the mass of the sample is shown. Note that in most cases contamination resulted in mass increase, but in some cases mass loss was observed. It is evident that changes of such order of magnitude are not negligible during quantitative calculations. Furthermore, it can be seen, that the total mass change during analysis depends on the individual, not controllable conditions of analysis so, it is advisable to check the mass change during each analysis by recording the transmitted beam current (i.e. voltage of the first step of

scanning amplifier) versus the analysing time. In other words, this means that the contamination (or mass loss) can not be regarded as instrumental parameter (impossible to give a standard contamination rate) and as it is not negligible, it is indeed necessary to determine the actual mass change in the case of each analysis.

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Microwave Method for Determining Dielectric Parameters of Living Biological Objects. III. Study of Water Binding in Frog Nerve

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Dielectric constant and loss of frog nerve was determined at 6.3 GHz frequency and 20–50 °C temperature by a microwave cavity resonator measuring method developed by us. As a result of temperature denaturation the dielectric constant of frog nerve decreases, while its dielectric loss increases. According to our measurements the volume of bound water in nerve is ~ 0.44 ml/l ml dry material. The activation energy of water in the nerve is 16.8 kJ/mol.

Introduction

In recent years the use of different microwave measuring methods (measuring the standing wave ratio, damping factor, reflection coefficient, impedance; cavity resonator measurements; TDR, etc.) have become more and more widespread in biological research work. Measuring with cavity resonator technique is advantageous because of its sensitivity and relative simplicity. Here the choice of the type of cavity resonator and its resonant mode is governed by the shape of the probe, the definiteness of its interaction with the electromagnetic field, and by the complexity of the evaluation procedure. Considering the above-mentioned reasons the use of circular cavity resonator operating in the TM_{010} mode is advantageous for measuring biological probes (liquids, living objects). Kraeft et al. (Kraeft, 1965; Kraeft, Gerdes, 1965) used this type of cavity resonator for measuring water and diluted electrolyte solutions, and we also constructed a cavity resonator of this type for measuring biological probes (Misik et al., 1978). The dielectric parameters of certain body tissues at different frequencies have already been determined, (some dielectric constant values can be seen in Table 1), but we could not find any data in literature about nerves.

The cavity resonator method elaborated by us is definitely convenient for the measurement of cylindrical biological probes. We have determined the dielectric parameters of frog nerves, so we were in a position to study the water binding of biological probes with high (appr. 80%) water content.

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Table 1

Relative permittivity of body tissues at $t = 37\,^{\circ}C$
(Almássy, 1973, p. 964)

Tissue	Frequency (GHz)					
	0.1	0.2	0.4	1.0	3.0	8.5
Muscle	71–76	56	52–54	49–52	45–48	40–42
Liver	76–79	50–56	44–51	46–47	42–43	34–38
Kidney	87–92	62	53–55	—	—	—
Skin	65	—	46–48	43–46	40–45	36
Fat tissue	—	4.5–7.5	4–7	5.3–7.5	3.9–7.2	3.5–4.5

Materials and method

The frogs *(Rana esculenta)* were decapitated, the myelon was disintegrated, then the nervus ischiadicus was dissected at all length. The dissected nerve was taken under Ringer solution into a glass capillary with an inner diameter just enough to be completely filled by the nerve, and there was no Ringer solution left between the nerve and the inner wall of the capillary. (This is a very important fact because even a small amount of Ringer solution may cause significant errors in measurements.)

The two ends of the capillary were closed by paraffine, then the capillary was put into a cavity resonator type TM_{010} and the ε' dielectric constant and the ε'' dielectric loss of the probe were determined at 6.3 GHz frequency at 20–50 °C temperature. Some nerve dissects were killed by a stay of 1 hour at 60 °C, and the change in dielectric parameters was measured. The ε' and ε'' values of dry material content of frog nerves were determined after having dried the nerve at 105 °C for 24 hours.

Results

1. The ε' and ε'' values of the measured frog nerves are shown in Tables 2 and 3. (ε' and ε'' average values and the standard deviation of the mean value are given at different temperatures.)

2. The water content of frog nerves was 77–83%, so the measured ε' values were compared to the relevant values of a gelatine solution of 20% concentration (Fig. 1). The measured values of dry material in the nerves were $\varepsilon' = 2$–5 and $\varepsilon'' = 0.1$–0.4, which are similar to the relevant values of dried gelatine. There is a significant difference between the temperature dependency of ε' values of nerves and 20% gelatine solution – in spite of the same water content and the same ε' value of dry material.

Table 2

ε′ values of frog nerves between 20–50 °C

20 °C	25 °C	30 °C	35 °C	40 °C	45 °C	50 °C
51.2	51.0	51.9	51.5	50.5	50.4	49.0
50.5	51.9	52.2	52.2	52.2	51.6	51.0
47.7	48.4	49.5	49.7	48.2	48.3	46.9
49.5	49.6	50.6	49.8	48.7	49.0	48.0
48.2	49.0	49.7	49.6	49.7	49.3	48.2
51.3	51.0	50.8	51.9	51.9	51.2	50.5
47.5	48.1	47.9	48.3	47.8	47.5	46.7
49.2	49.4	49.9	50.2	49.2	48.9	48.0
49.5	49.3	49.0	49.1	49.1	48.1	47.0
52.0	52.1	51.6	51.6	51.4	50.7	49.6
50.7	51.2	51.1	51.0	50.6	50.2	49.1
52.2	52.1	52.1	51.8	51.3	50.6	49.9
55.1	55.1	55.2	55.1	54.6	54.0	53.4
53.7	54.1	54.3	53.9	53.6	53.0	52.3
49.2	49.5	49.3	49.9	48.7	48.0	48.1
53.5	54.0	54.1	53.7	52.8	52.3	50.8
49.5	50.1	50.3	50.7	49.5	49.6	48.2
59.9	51.1	51.1	51.0	50.8	50.4	49.6
51.8	52.2	52.0	52.0	51.5	50.7	49.8
45.6	47.0	47.1	47.4	47.5	47.0	46.8
44.9	45.6	45.7	46.2	45.9	46.2	45.4
52.8	53.1	53.1	52.9	52.7	52.1	51.6
54.1	55.6	55.1	54.7	54.6	53.1	52.5
48.3	49.5	49.7	49.4	49.7	49.3	48.6
52.6	53.1	52.7	52.8	52.5	52.1	51.5
50.8 ± 0.6	50.9 ± 0.5	51.0 ± 0.5	51.0 ± 0.4	50.6 ± 0.5	50.1 ± 0.4	49.3 ± 0.4

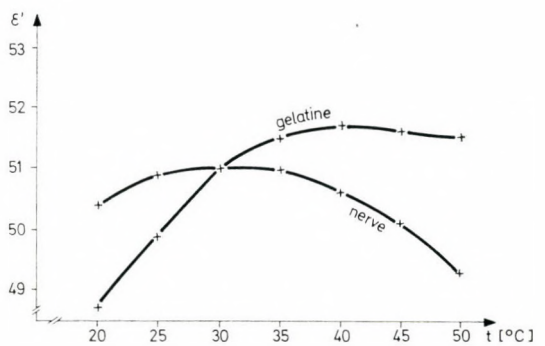

Fig. 1. Temperature dependence of dielectric constant of frog nerve and of 20% gelatine solution

Table 3

ε″ values of frog nerves between 20–50 °C

20 °C	25 °C	30°	35 °C	40 °C	45 °C	50 °C
21.2	19.4	18.4	16.6	15.2	14.1	13.1
23.0	20.8	19.1	17.4	16.5	15.3	14.5
21.5	20.1	19.4	17.1	16.2	15.0	14.2
21.1	19.1	18.3	16.1	15.4	14.3	13.3
22.0	18.4	17.2	16.0	14.8	14.0	13.1
20.9	19.0	18.2	16.4	14.9	14.3	13.4
19.3	17.9	16.1	15.2	13.8	13.2	12.3
21.6	20.0	18.8	17.2	16.5	15.1	14.4
19.7	18.6	17.0	15.6	15.1	14.5	13.6
20.7	19.9	17.7	16.7	15.8	14.8	14.5
20.1	19.3	17.6	16.7	15.4	14.5	14.1
21.1	19.0	17.5	16.2	15.7	15.0	14.0
22.0	19.9	18.3	17.0	15.9	15.0	14.1
21.6	19.6	17.9	16.6	15.7	14.6	13.6
19.3	18.1	16.7	15.5	14.4	13.4	13.2
21.7	20.6	18.3	17.0	16.1	15.0	14.9
20.5	19.0	17.6	16.3	15.2	14.2	13.1
20.3	18.6	17.2	16.1	15.1	14.1	13.3
21.1	19.4	17.9	16.7	15.8	14.7	14.2
19.7	18.0	16.6	15.4	14.1	13.4	12.7
19.3	17.9	16.1	14.9	13.8	13.1	12.1
21.5	19.4	18.0	16.8	15.6	14.8	14.1
22.3	20.4	18.7	17.5	16.5	15.3	14.4
19.5	17.9	16.5	15.3	14.4	13.2	12.5
21.0	19.4	18.0	16.9	15.7	15.0	13.9
20.9 ± 0.2	19.2 ± 0.2	17.7 ± 0.2	16.4 ± 0.1	15.3 ± 0.2	14.4 ± 0.1	13.6 ± 0.2

3. In the case of some probes after the dielectric parameters had been determined the nerves were killed by a stay at 60 °C for 1 hour and the ε' and ε'' values of the killed nerves were determined again (Table 4).

The ε' values of temperature denatured nerves were lower, their ε'' values were higher than those of live nerves. It is a small change, but the ε' decrease and ε'' increase at $P = 0.1\%$ level is significant when mean values are compared by difference-method, and the *t*-test gives the same result.

4. According to Szkanavi's (1973) method the ε' value of the mixture is:

$$\varepsilon' = p_1 \cdot \varepsilon'_1 + p_2 \cdot \varepsilon'_2 , \qquad (1)$$

where p_1 and $p_2 = (1 - p_1)$ is the volume ratio of the two components.

This formula offers a possibility for the estimation of the quantity of bound water in the nerves. (There are various approaching formulas to calculate dielectric constant of mixtures [Tinga et al., 1973], but in the absence of a formula correctly applicable to living systems we have chosen the simplest approximation.)

Table 4

Dielectric parameters of frog nerves a) *before temperature denaturation and* b) *after temperature denaturation (1–4, frog nerves)*

ε' and ε'' \ °C		20	25	30	35	40	45	50
ε'	1. a)	49.2	50.2	49.3	50.3	50.2	49.7	48.4
	b)	48.8	48.9	49.0	48.8	49.3	48.7	48.3
	2. a)	52.2	52.6	52.7	52.6	52.1	51.5	50.9
	b)	51.8	52.2	52.2	52.2	52.0	51.7	51.2
	3. a)	49.5	50.2	50.5	50.5	50.1	49.4	48.7
	b)	49.3	49.7	50.1	49.9	49.0	49.1	48.6
	4. a)	50.4	51.3	51.7	51.1	49.3	49.2	47.8
	b)	50.2	50.6	49.5	50.0	49.1	48.8	47.9
ε''	1. a)	20.7	19.0	17.6	16.6	15.5	14.5	13.6
	b)	21.6	20.1	18.3	16.9	16.1	15.0	14.0
	2. a)	21.0	18.9	17.3	16.0	14.9	14.0	13.1
	b)	21.4	19.7	18.3	16.8	15.6	14.4	13.5
	3. a)	21.5	20.1	18.4	17.1	16.2	15.0	14.2
	b)	22.3	20.6	18.8	17.8	16.8	15.3	14.5
	4. a)	20.9	19.4	17.8	16.4	15.6	14.5	12.9
	b)	21.1	20.4	18.2	17.0	17.4	15.0	13.3

Because of water binding, relation (1) should be written out – as a first approximation of the complex system – for three components – "free" water, „bound" water and the dry material of the nerve:

$$\varepsilon' = p_1 \cdot \varepsilon'_1 + p_2 \cdot \varepsilon'_2 + p_3 \cdot \varepsilon'_3 . \tag{2}$$

Further on the following designations are used:

p_{ww} is the volume ratio of the total (= free + bound) water;

p_{fw} and ε'_{fw} are the volume ratio and ε' value of free water;

p_{bw} and ε'_{bw} are the volume ratio and ε' value of bound water;

p_d and ε'_d are the volume ratio and ε' value of dry material in nerve;

ε'_m is the measured ε' value of the mixture. Thus:

$$\varepsilon'_m = p_{fw} \cdot \varepsilon'_{fw} + p_{bw} \cdot \varepsilon'_{bw} + p_d \cdot \varepsilon'_d , \tag{3}$$

where $p_{fw} + p_{bw} + p_d = 1$, and

$$p_{fw} = p_{ww} - p_{dw} .$$

So relation (3) can be written in the following form:

$$\varepsilon'_m = (p_{ww} - p_{bw}) \cdot \varepsilon'_{fw} + p_{bw} \cdot \varepsilon'_{bw} + p_d \cdot \varepsilon'_d . \tag{4}$$

In this relation values ε'_m, p_{ww}, ε'_{fw} and ε'_d are given or are measurable, while the values of p_{bw} and ε'_{bw} are unknown.

From Table 2 (t = 30 °C) $\varepsilon'_m = 51$; considering the 80% water content of the nerve, $p_{ww} = 0.8$; the dielectric constant of free water (t = 30 °C) $\varepsilon'_{fw} = 70.1$ (Misik et al., 1978); the ε' value of dry material in the nerve is $\varepsilon'_d = 3.5$; and one may presume that $\varepsilon'_{bw} = 5$ (Pennock, Schwan, 1969). So calculating from relation (4), from the data measured at 30 °C the quantity of bound water in the nerve is 0.44 ml/1 ml dry material. As it is going to be demonstrated in a following paper, adapting other approaches instead of the simplest formula used here would make us significant change in this value.

5. The activation energy of water can be calculated from the temperature dependency of dielectric parameters (Misik, Masszi, 1981). Using our measured data the activation energy of water is 18.1 ± 1.3 kJ/M, which agrees with data in literature (Sandus, Lubnitz, 1961; Pottel, Lossen, 1967). On the basis of the calculating method used at the activation energy of water it is possible to calculate also the activation energy of water in the nerve, but at the ε'' values of the nerve the loss represented by the all-ion-content of the nerve should also be taken into consideration (this is 3.2–5.5 between 20–50 °C). These values corrected and plotting log. $(\varepsilon' - \varepsilon_\infty)/\varepsilon''_{corr}$ against $1/T$, the activation energy of whole (= free + + bound) water in the nerve will be $E = 16.8 \pm 1.7$ kJ/mol.

Discussion

In differentiated tissues water is distributed among extracellular and intracellular areas. The properties of extracellular water can be described on the basis of the properties of pure water and electrolyte solutions, intracellular water, however, has at least two fractions, the characteristics of which are rather different.

There are two hypotheses concerning the characteristics of intracellular water. One says that the main bulk of intracellular water (= so-called "bulk water") is similar to a diluted salt solution – by reason of its viscosity, hydrogen binding rate, self-diffusion, etc. – and only a smaller part of water has – because of its strong interaction with macromolecules – different properties (= bound water) (Abetsedarskaya et al., 1968; Finch et al., 1971; Burke et al., 1974; Stout et al., 1977 etc.). The other hypothesis assumes that owing to the concentric layers (= "multilayer") of the oriented water molecules, intracellular "bulk" water is more structured than water in diluted salt solutions (Ling, 1965; Cope, 1969; Hazlewood et al., 1969; Masszi et al., 1976; Chang, Woessner, 1977, etc.). According to certain ESR data (Belágyi, 1975) the volume of bound water is bigger than the value presumed by the first hypothesis, about 4–5% of the whole water con-

tent, "bulk" water, however, does not show the high level order presumed by the second hypothesis.

The estimation following the second formula seems to confirm Belágyi's opinion. We found 0.44 ml/1 ml dry material as the volume of bound water in the nerve, which represents about 11 per cent of the whole water content. This is remarkably higher than the value presumed by the first hypothesis. (In literature generally a value of 0.2–0.4 g/1 g dry material is given as the volume of biological bound water.) The value of 16.8 kJ/mol as activation energy of the whole water in the nerve (which is close to the value 18.1 kJ/mol got for pure (= free) water), however, contradicts the fact that the order of "bulk" water would be induced by a more explicit H-bridge system. It is remarkable that the activation energy of the whole water in the nerve is less than that of pure water. Harvey, and Hoekstra (1972) stated that the activation enthalpy and entropy of bound water can also be a negative value; as they wrote; "It is usual in that it moves to lower frequencies as the temperature is raised (τ increases). This is because the activation enthalpy is negative." As the activation energy of dipole rotation in ice is 54.6 kJ/mol (Auty, Cole, 1952), our results, confirming the data of Pócsik (1969), Masszi (1970) and others, contradict the so-called "iceberg" hypothesis (Szent-Györgyit 1957), according to which "Many aspects of protein behaviour can be interpreted in terms of frozen water of hydration" (Klotz, 1958).

The temperature dependency of the dielectric constant of the nerve differs both from that in pure water and electrolyte solutions (Misik, Masszi, 1981) and from that in gelatine solution of a similar water content (= 20% concentration). So protein-water interaction alters the structure and dielectric properties of water, and the biological structure, the ions bound to proteins take also their part in this change.

The role of bound ions is manifested also in our experiments with the temperature denaturation of the nerve. As an effect of temperature denaturation the ε' values of the nerve decreased, its ε'' values increased. This effect resulted probably from the joint action of ions and water released from bound state.

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The Importance of Changes in ATP and ADP Concentrations in the Development of the Phasic Superprecipitation of Actomyosin

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It has been shown previously that at physiological K^+-concentrations the development of superprecipitation has a phasic character. Studying the reason of this effect, by the use of beta-^{32}P-ADP ATP-regeneration from ADP was detected during the first phase of superprecipitation. Myokinase activity of actomyosin may be responsible for the appearance of this first phase of turbidity change of actomyosin suspension. Myokinase contributes to the stabilization of an ATP/ADP ratio within narrow limits. During the first phase of superprecipitation the ATP/ADP ratio is between these limits, while during the second phase the ratio has a low value.

This phasic evolution of superprecipitation is, probably connected with the physiological functional state of the muscle (contraction or rigour).

Introduction

In our previous experiments carried out on rabbit skeletal muscle actomyosin superprecipitation was found to have a phasic character under physiological conditions (Csabina, et al., 1980; Szöőr, et al., 1982). After addition of ATP to the suspension, clearing was first followed by a moderate (first phase), then by an intensive (second phase) increase in turbidity. Parallel with this optical change, ATP is splitted to ADP by myosin-ATPase which is activated by actin present in the suspension (Straub, 1942; Eisenberg, Moos, 1968; Eisenberg, Moos, 1970). Important variables in the composition of the medium are therefore the concentrations of ATP and ADP. There have been observations proving that an increase in the ADP concentration decreases the activity of myosin ATPase (Nanninga, 1962), and increase the turbidity of actomyosin (Maruyama, Gergely, 1962). Similarly the activity of enzymes regenerating ATP from ADP have relaxing effect on myofibrillar peparation (Lóránd, 1953), and on glycerinated (Moos, Lóránd, 1957) as well as on intact muscle (Dydynska, Wilkie, 1966).

On the basis of these data it was assumed that the phasic character of superprecipitation may be caused by intermittent changes in the ATP/ADP ratio. The aim of our experiments was to study this question.

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Materials and methods

The preparation of natural actomyosin and the method of superprecipitation have been reported in our previous paper (Szöőr et al., 1982).

For ATP determination in the course of superprecipitation 20 μl samples were taken from the reaction mixture, which were added to 180 μl of 10% trichloro--acetic acid solution, in order to stop any further reaction. Then luciferin-luciferase enzyme (LKB product) in 0.1 M TRIS-acetate-EDTA buffer (pH: 7.75) was added to the samples (Myhrman, et al., 1978). After this the ATP-concentration-dependent bioluminescent light intensity was measured with a Packard TRI-CARB 3320 liquid-scintillation counter. The calibration curve fitted the following function: $\lg [ATP] = a_0 + a_1(\lg I) + a_2(\lg I)^2$.

The temporal changes in the distribution of radioactivity among the different products were determined by chromatographic method, from reaction mixtures containing labelled nucleotides (Abbot, Leech, 1973). The 2 μl samples taken from the reaction mixture at one-minute (sometimes at half-minute) intervals were placed on PEI-cellulose thin layer chromatographic plates (MERCK) which, in turn, at the place of application, had previously been treated with 5μl 10% tri--chloro-acetic acid for the establishment of enzyme reaction. The chromatogram was developed in 0.4 M KH_2PO_4 solution (RF values: ATP: 0.14; ADP: 0.39; P_i: 0.65). The individual components were autoradiographically localized, then cutting out the corresponding chromatogram areas, their radioactivity was measured in a liquid scintillator (5.0 g PPO, 0.25 g POPOP in 1 litre of toluene). The ratio of radioactivity for a given component was calculated as the percentage of the total radioactivity of all constituents (10 000 cpm). The labelled compounds were produced in our laboratory using ^{32}P-phosphoric acid (Isotope Institute of the Hung. Acad. Sci.), (Beutler, Guinto, 1976; Rosett et al., 1970).

The composition of our reaction mixture was the following: 1 g $\cdot$ dm^{-3} actomyosin, 0.140 M KCl, 0.001 M $MgCl_2$, 0.0001 M $CaCl_2$, 0.020 M Tris-HCl buffer (pH: 7.0), 0.001 M ATP. The reaction was started with the addition of ATP. The deviations from the control values are denoted in the figures.

Results

Superprecipitation evoked by ATP is modified by the presence of ADP in close relation with its concentration. In addition, superprecipitation can be elicited not only by ATP, but with ADP, too (Fig. 1).

The phenomenon refers to the fact that the actomyosin preparation we used is capable of synthesizing ATP from ADP, i.e. it shows myokinase activity too. Myokinase activity of contractile proteins have been recently published by Belliveau, et al. (1981) and Shriver and Sykes (1981).

The changes in the ATP-ase activity of the actomyosin preparation, taking place during the development of superprecipitation, were followed by two methods

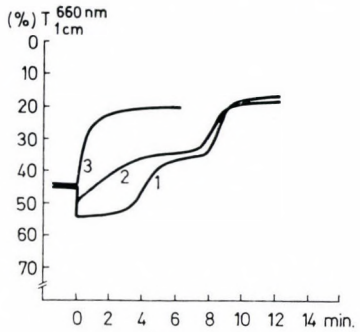

Fig. 1. Effect of ADP on superprecipitation. 1. Control (1 mM ATP induced superprecipitation). 2. 0.5 mM ATP + 0.5 mM ADP induced superprecipitation. 3. 1.0 mM ADP induced superprecipitation

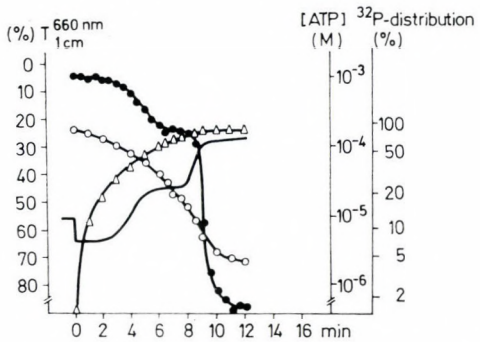

Fig. 2. Changes of ATP and P_i levels during superprecipitation. ●●●● ATP concentration determined by luciferin-luciferase. Distribution of radioactivity between ATP (○○○○) and P_i (△△△△) fractions in the case of gamma-^{32}P-ATP induced superprecipitation. —— superprecipitation curve

independent of each other. By the use of luciferin-luciferase the actual ATP concentration was measured, by the application of gamma-^{32}P-ATP the distribution of radioactivity between the P_i and ATP fractions was determined (Fig. 2).

The ATP level, as determined by bioluminescence, synchronously with superprecipitation, changes step-wise as a function of time. The ATP level that can be measured by the application of gamma-^{32}P-ATP does not show phasic changes, since in this case regenerated ATP cannot be detected.

To resolve the contradiction between the two measurements, beta-^{32}P-ATP was also used. In this case ATP remains radioactively detectable even after synthesis from ADP. The curve obtained when using labelled ATP at the beta position, similarly to the results of measurement with the luciferin-luciferase system, is of phasic character and the phases show synchronism with superprecipitation (Fig. 3). Under such conditions radioactivity appearing in the P_i fraction is the result of the decomposition of that ATP which was newly synthetized from ADP.

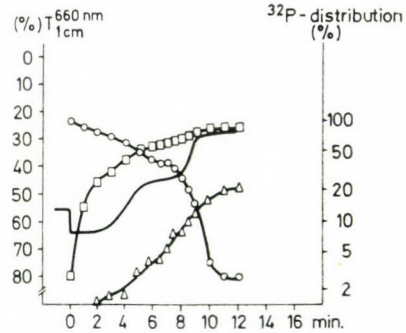

Fig. 3. Distribution of radioactivity during beta-^{32}P-ATP induced superprecipitation. ○○○○ ATP; □□□□ ADP; △△△△ P_i; —— superprecipitation curve

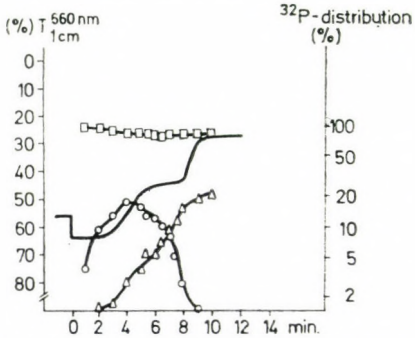

Fig. 4. Distribution of radioactivity between ATP, ADP and P_i in the control reaction mixture containing 1 μM beta ^{32}P-ADP. ○○○○ ATP; □□□□ ADP; △△△△ P_i; —— superprecipitation curve

The fact of ATP-regeneration is shown from results presented in Fig. 4: ATP is formed from beta-^{32}P-ADP, which later, due to ATP-ase activity, is decomposed with the release of radioactive orthophosphate.

Discussion

Our results show that natural actomyosin prepared from rabbit skeletal muscle possesses myokinase activity. Myokinase or adnylate kinase (E.C.2.7.4.2) synthesizes ATP from ADP:

$$2\,\mathrm{ADP} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{ATP} + \mathrm{AMP}$$

$$K = \frac{k_1}{k_2}\,\frac{[\mathrm{ATP}][\mathrm{AMP}]}{[\mathrm{ADP}]^2} = 0.442.$$

The phasic superprecipitation of actomyosin suspension exhibited in the presence of 0.14 M KCl can be interpreted according to the following mechanism:

At the moment of starting the reaction, as a result of the relatively high (1 mM) ATP concentration, the dissociation of actomyosin results in a clearing of the solution (clearing phase). The activation of actin is not manifested on myosin ATP-ase (Eisenberg, Moos, 1968; Eisenberg, Moos, 1970), thus ATP decomposition is slow (the average rate of ATP decomposition is 1.1 mol · $dm^{-3}min^{-1}$) in the first 2 minutes. As a result of the decrease in ATP concentration the association of actin and myosin gradually takes place. The turbidity of the reaction mixture increases and superprecipitation starts. ATP-ase activity is also enhanced. The mean rate of ATP decomposition is 1.59 mol · $cm^{-3}min^{-1}$ in the 3rd to 4th minutes and 3.03 mol · $dm^{-3}min^{-1}$ in the 5th to 6th minutes. As a result of the enhancement of ATPase activity the concentration of ADP increases quickly, making the synthesis of ATP by myokinase possible. As a result of this latter process the rate of decrease in ATP concentration is moderated (in the 7th to 8th minutes it is 1.32 mol · $dm^{-3}min^{-1}$); the rate of association between actin and myosin decreases, i.e. the first phase of superprecipitation takes place.

Further decrease in the ATP level involves the enhancement of actin activation, the second phase of superprecipitation evolves and, in the mean time, ATP decomposes quickly. Myokinase activity is able to keep up with the extremely rapid ATP decomposition only in a transient period (the average rate of ATP decomposition in the 9–10th minutes is 172.4 mol · $dm^{-3}min^{-1}$).

The parameters of the first phase of superprecipitation may be different in the particular preparations, or can be modified by changing the conditions (Csabina, et al., 1980; Szöőr, et al. 1982). Its regular occurrence and the correlation with myokinase activity, on the other hand, indicates that it corresponds to a physiologically existing phase of muscular contraction which is regulated by more than one factor. Characteristics of this phase may be connected with the degree of activation of contractile proteins. There are several data to suggest that the activation is related to the phosphorylation-dephosphorylation of the phosphorylizable light chain (LC_2) of the myosin head, too (Kasman et al., 1980; Kakol et al., 1980; Bárány, Bárány, 1980; Adelstein, 1980). Recently, this latter process, as a phenomenon participating in the regulation of contraction, has been proved to be valid also in the case of the skeletal muscles (Stull, Blumenthal, 1980; Persechini et al., 1981).

The authors would like to thank Prof. A. Kövér, and Prof. E. Varga for helpful discussions and for criticism of the manuscript.

The authors are greatful to Miss K. Szegedi, Mrs. E. Szakáll and Mrs. R. Tóth for skilful laboratory assistance.

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BOOK REVIEWS

The Role of Intercellular Signals: Navigation, Encounter, Outcome by John G. Nicholls (ed.) (Report of the Dahlem Workshop on the Role of Intercellular Signals: Navigation, Encounter, Outcome. Berlin, 1979, March, 12 to 16). Life Sciences Research Report 14. Verlag Chemie, Weinheim, New York 1979, 309 pp.

This book reports on the Dahlem workshop directed to one of the most exciting fields of recent research: the variety of cell-cell communication, and provides general coverage of this topic. Each chapter has been prepared by an expert or a group of experts. They discuss questions such as chemical signaling in the nervous system, chemically activated membrane channels, interactions among cells of the immune system, control of cell movement, cellular migration and axonal outgrowth in the peripheral nervous system, the primary process involved in the encounter of cells, functional differentiations of the intercellular junctions, extracellular regulation of neurons, cell-cell interactions within the early embryo, mechanism of spermovum interaction. The last four chapters form a framework for the discussion of "Signal operation and reception", "Cellular navigation", "Cell-cell encounter", "Consequences of signal reception".

In recent years there has been a rapid advance in the study of "molecular physiology" and the presented topics are part of this trend.

The limited extent of the book allows only a short comprehensive treatise to be given and this seems to be unjust regarding the significance of these major subjects. However each chapter concludes with an abundant collection of references which help the reader build up a detailed knowledge of the data and makes available the original papers for a thorough analysis.

This volume will be of great value to scientists, teachers and graduate students concerned with cell and neurobiology.

L. Boross

The Sarcoplasmic Reticulum. Transport and Energy Conversion by Leopoldo de Meis. 2nd volume of the Wiley series "Transport in Biological Sciences" edited by E. Eduard Bittar. pp XV and 163. J. Wiley and Sons Ltd., England, 1981

Relying on hi own results, the author conceives a comprehensive view of the present-day knowledge in this field.

The primary object of this monograph is to give an overall and comprehensive picture of the physical, chemical and functional characteristics of the sarcoplasmic reticulum (SR) and Ca, Mg-dependent ATPase. Chapter 1 discusses the structural localization of SR and its relationship with myofibrils and with the transversal system (T-system). The way the excitation of the surface of the fibrils leads to the activation of SR and then to that of the contractile system, furthermore the role of SR in bringing about relaxation are briefly touched upon.

Chapter 2 deals with the isolation of SR vesicles and the biochemical characteristics, role, distribution and asymmetric arrangement in membranes of its constituents (pro-

teins, lipids). A special emphasis is laid on the role of the so-called annular phospholipids (30 moles of phospholipid/mole monomeric ATPase) in maintaining the functional state of ATPase. Phospholipids may be replaced by amphiphilic (non-ionic) detergent molecules. They also play an important role in the formation of the ATPase oligomers necessary for transport.

Chapter 3 outlines the problem of active calcium transport. Calcium uptake is coupled with the hydrolysis of ATP (at a ratio of 2 : 1) in the course of which a phosphoenzyme of acylphosphate character is formed, which is detectable by ATP-ADP exchange measurements (in all these three processes $K_m = 0.1-2.0\ \mu M$). In the active site the same tripeptide (serine or threonine, phosphoaspartate and lysine) was identified as in the case of Na, K-ATPase. The saturation of the high-affinity calcium binding sites oriented towards the cytoplasm ($K_s = 0.1-2.0\ \mu M$) is a prerequisite of ATP-dependent phosphorylation. The maximal rate of ATP hydrolysis is limited by the increasing intravesicular concentration of calcium (reverse inhibition) and by the saturation of the low-affinity binding sites ($K_s = 1-2$ mM). The effect of detergents, ionophores and anions sequestring calcium can be interpreted by the suspension of reverse inhibition. In the course of the hydrolysis, high-affinity binding sites may turn into low-affinity ones and vice versa. Enzyme affinity for ATP is by several orders of magnitude higher than for other energy-yielding substrates. K_m values for the hydrolysis of MG-ATP are $1-3$ and $50-200\ \mu M$. The former value characterizes binding to the catalytic center, while the latter belongs to one of the activation steps.

Chapter 4 discusses the reversibility of the overall process of calcium transport. In the presence of EGTA, calcium slowly leaks out of vesicles filled with calcium; if ADP, Mg^{2+} and inorganic phosphate (P_i) are added to the medium, this process is suddenly accelerated and ATP is synthesized. In the absence of ADP, phosphoenzyme may be formed but this is followed neither by the enhancement of calcium outflux nor by ATP synthesis. Mg^{2+} is essential for P_i-dependent phosphorylation. The steady-state level of EP is a function of the extent of the calcium gradient. ATP synthesis is inhibited by the ATP or calcium content of the medium. Ca^{2+} bound to the high-affinity binding sites inhibits P_i-phosphorylation irrespective of the extent of the gradient. ATP hydrolysis supplies energy for ATP synthesis. According to recent data, however, the latter process is energized not by the calcium gradient but by the binding of ADP, P_i and calcium to different binding sites of the enzyme.

The ATPase can be phosphorylated also in the absence of a Ca gradient (with EP formation) in the presence of Mg^{2+} and 2—4 mM P_i, especially if the pH of the medium is adjusted to pH 6 instead of 7. (Chapter 5.). This can be proved by measuring the incorporation of ^{18}O-labelled H_2O into P_i. The enzyme forms a ternary complex with Mg^{2+} and P_i (E.P_i.Mg) and this is in equilibrium with E-P.Mg phosphoenzyme. When the pH is increased from 6.0 to 7.0 the equilibrium level of EP is suddenly decreased. Phosphorylation is proportional to the concentration of primary phosphate (depending on the pH). This pH-dependence is abolished in the presence of glycerol or other organic solvents. Depending on the Ca concentration of the medium, phosphorylation by P_i decreases and that by ATP increases. The choice of substrate is determined by the conformational change brought about by the binding of Ca. In the reaction cycle of ATP hydrolysis there also exists such a transitional enzyme form which can be phosphorylated only by P_i (Chapter 6). Accordingly, the inhibition of P_i-dependent phosphorylation (via Ca binding to high affinity binding sites) may be suspended by the addition of energy-yielding substrates to the medium (ITP, GTP, ATP, acetyl-P). The binding of substrates to the catalytic site promotes the establishment of the P_i-reactive conformation (with ATP at μM, with other substrates at mM concentrations). The saturation of the low-affinity binding sites with calcium increases the steady-state EP level in ITP- and P_i-dependent phosphorylation. In the case of leaky vesicles, the level of EP is high only if, in order to saturate low-affinity calcium binding sites, the Ca^{2+} concentration of the medium is increased to ca. 20 mM (in the presence of ITP). When a calcium gradient is present, the ATPase catalyzes also the ATP-P_i exchange. With leaky vesicles, the increase in the calcium concentration of the medium

activates ATP synthesis and at the same time inhibits ATP hydrolysis. In order to keep up ATP-P_i exchange, it is necessary to saturate the low-affinity binding sites. The exchange does not take place in the absence of ADP however, the amount of ADP originating from ATP hydrolysis is sufficient (with IDP, much higher concentrations are needed). Binding of the nucleotides to the regulatory (non-hydrolytic) sites of the enzyme enhances the breakdown of ATP instead of its synthesis.

Studies on the sequence of partial reactions (Chapter 7) revealed that the enzyme occurs in two different functional states (E and $^+$E). In form E, the calcium binding site looks outwards (K_s for calcium = 0.1—0.2 μM at pH 7.0), while in form $^+$E it is oriented inwards (K_s = 1.0—2.0 mM at pH 7.0). After calcium binding, form E can be phosphorylated by nucleotides or other energy-yielding substances and form $^+$E can be phosphorylated by P_i. The conversion of $^+$E into E is speeded up by ATP, GTP, ITP. From the various phosphoenzymes only $Ca_2 \cdot E \sim P$ transfers P_i to ADP. The chapter discusses the distribution of $Ca_2 \cdot E \sim P$, $Ca_2 \cdot {}^+E \sim P$ and $^+E—P$ in steady state, determined by a simultanous measurement of the EP level and the P_i-HOH and ATP-P_i exchanges. $^+$E-P is converted into $Ca \cdot E \sim P$ when calcium is bound by the low-affinity binding sites. From E—P no ATP can be synthesized under the effect of ADP. It seems probable that it is the binding of ADP to the phosphoenzyme that brings about the conversion of low-affinity (low energy) binding sites into high-affinity (high energy) ones. A better understanding of the intermediate reactions of the catalytic cycle of ATPase is facilitated by studies on transient kinetics (Chapter 8). Starting from different points of the reaction series, the velocity of phosphorylation induced by either ATP or P_i can be measured. The interconversion of the two different conformational variants of the enzyme (E and $^+$E) and therefore the susceptibility of the enzyme to phosphorylation by P_i or ATP are influenced by a number of factors. In both cases the interconversion is the rate-limiting step of the overall reaction. The chapter describes what conclusions can be drawn from the kinetic analysis of EP formation and breakdown and of the changes in the ADP sensitivity of EP regarding partial reactions, kinetic constants, interactions and time constants.

Chapter 9 discusses the temperature dependence of ATPase function. Temperature dependence is determined by the phase transition of protein-bound lipids which is different from that of the surrounding lipids (shells). In the case of DPL-ATPase complexes the regional fluidity of annular lipids is sufficient for some preservation of ATPase activity (even if the extraannular lipids are crystallized). Since activated and solubilized ATPase freed of lipids by detergent treatment ($C_{12}E_8$) exhibits a temperature dependence identical with that of SR ATPase, it seems probable that the break observed at 20 °C in the Arrhenius plot is not caused by lipid-protein interaction but by an intrinsic temperature-dependent change of the polypeptide chain.

Chapter 10 describes the effects of cations, pH and cAMP. The apparent affinity to strontium is about one order of magnitude lower than to calcium. Calcium dependent phosphoenzyme formation occurs even in the absence of Mg; however, the rate of hydrolysis decreases to 0.01. Mg enhances the $^+E—E$ and AFP-sensitive-ADP insensitive phosphoenzyme conversions and also the hydrolysis of the latter. Physiological concentrations of K^+ and N^+ decrease the Ca^{2+} affinity of the high-affinity binding sites. In the course of contraction the inhibition is suspended by the increasing Ca-level and Ca-uptake increases. At this Ca^{2+} concentration K^+ already increases the activity of the pump. On raising the pH, the Ca^{2+} affinity of the binding sites increases. ATP synthesis is optimal at pH 6, if the low-affinity binding sites are saturated. Also, it is only in this case that phosphate can be transferred from EP to ADP. The proton gradient plays no direct role, it is only a concomitant phenomenon. cAMP increases Ca-transport by the phosphorylation of the 22 000 dalton protein of heart SR vesicles and by the modulating effect of the phosphorylated protein. Ca, Mg-ATPase may synthesize small amounts of ATP even in the absence of Ca gradient (Chapter 11). In the presence of EGTA and Mg at pH 6, leaky vesicles can be phosphorylated first by P_i and then by the addition of ADP and Ca; on raising the pH to 7.4 (in order to

enhance the low-affinity calcium binding sites) ATP is synthesized. The energy used for the synthesis is that produced by the binding of Ca, Mg and substrates to the enzyme, i.e. the energy of binding is converted into chemical energy. It follows from the pH-dependence of the affinity of the binding sites that ATP can be synthesized also in the absence of Ca gradient, if the pH of the medium is acidic (the Ca affinity of the high-affinity binding states is lower) and at the same time the pH of the intravesicular fluid is slightly alkaline) the Ca affinity of the low-affinity binding sites is higher). The essential factor is not the proton pump but the asymmetric modification of the affinity of the binding sites. If t_0 is lowered from 30 °C to 0 °C, the Ca concentration necessary for ATP synthesis is decreased. The affinity of the high-affinity binding sites is not affected by changes of t_0. Similarly, in the case of intact vesicles it is the difference between the affinities of the two classes of binding sites that determines the extent of Ca gradient necessary for ATP synthesis. The foregoing, however, regards only the transfer of P_i from EP to AFP, since the enzyme itself is not phosphorylated at 0 °C or pH 8. Ca is needed for the conversion of the low-energy phosphoenzyme into the high-energy one. The extent of the organization of water at the catalytic site or in its immediate neighbourhood may play an essential role in energy conversion. The latter problem is covered in Chapter 12. The low and high energy forms of the enzyme may be correlated with the modification of the accessibility of the catalytic site for water. The formation of EP is favored in hydrophobic surroundings (e.g. in the presence of DMSO); however, EP will transfer P to ADP only if the hydrophobicity of the medium is decreased, in the present example by the addition of water, i.e. by dilution of DMSO. Thus acylphosphate can be formed spontaneously at the catalytic site, in a hydrophobic microenvironment. Accordingly, the energy-consuming process is not the formation of acylphosphate but the entrance of the reactants (P_i) into the microenvironment. Once acyl-P is formed, further energy is needed for the translocation of acyl-P into a hydrophilic (aqueous) environment, since it is only under these conditions that the high-energy form of acyl-P is established. As a conclusion, the quality of the phosphoenzyme depends on the extent of solvatation. The saturation by Ca of the low-affinity binding sites is necessary for the change in the character of the microenvironment and of the catalytic site, as the conformational change at this point results in the above-mentioned change in solvatation and permits the establishment of the high-energy form of the phosphoenzyme and, if AFP is present, the synthesis of ATP is possible.

A. KÖVÉR

Histologie und Histopathologie by H. Knoche. Kurzlehrbuch für medizinisch-technische Assistenten. Unter Mitarbeit von K. Addicks, H. Themann, K. M. Müller. Springer-Verlag, Berlin, New York 1980

This book was written for technicians employed in health service. Its authors are professors working in different fields of medicine in the FRG. H. Knoche was the director of the Institute of Anatomy at the University of Münster; he died shortly after the manuscript of this book had been put to press. K. Addicks is a specialist in electron microscopy at the Institute of Anatomy of Köln; H. Themann is a cytobiologist at the University of Münster, and K. M. Müller is a pathological anatomist at the same University.

This book of 347 pages is amply illustrated: it contains 174 Tables each of which is made up of 2—4 pictures. After a short and general introduction on cell morphology, the reader is introduced to normal hystological structure by the description of basic tissues (epithelial, connective and solid tissues, nervous tissue) and tracts (lymphoid, circulatory, respiratory and urinary systems etc.). Out of the 347 pages of the book the authors assigned 298 to this part, thus only 35 pages remained for histopathological problems. Accordingly, from the 174 Tables 160 illustrate the part on normal histology and 14 serve as illustration to chapters on histopathology.

The description of normal histology is clear and didactic, although it often seems to go into details deeper than needed, considering the knowledge necessary for the work of assistants. For this reason, however, medical

students, physicians and biologists with special training in fields other than histology will also find the book useful. A special merit of the book is that the description of structure is always accompanied by references to function, and, wherever possible, also to the biochemical, molecular organization of the histological structure. Throughout the book the most modern information is compiled. A critical analysis of the chapters hardly reveals any debatable point or deficiency worth mentioning (e.g. the concept of "amitosis" should not have been expounded in such a self-evident way: more emphasis should have been laid on the contractile system of the cell; the origin of the reticulin fibres is not described clearly; no mention is made of the APUD system, of myofibroblasts; in connection with normal cellular turnover, the phenomenon of apoptosis would have been worth mentioning; in the chapter on the lungs the concept of lobulus is mentioned only at the end of the chapter in small characters, whereas its knowledge is essential for the understanding of the normal organization of the lungs).

The information summarized on the 35 pages that deal with histopathology, is adequate but is restricted to the very essence of some chapters of general pathology (degenerations, necrosis, circulatory troubles, inflammations, tumors) and hardly demonstrates the significance of histopathology in medicine.

The illustrations of the book, made up exclusively of drawings, merit particular attention. The numerous black-and-white drawings simplifying and accentuating the essentials excellently correspond to the level at which technical assistants should be acquainted with normal histology. These drawings and their legends alone are nearly sufficient for conveying the essentials of histology. The question still arises whether it would not have been worth including 10—15 micrographs as well, in order to give the students more realistic visual experience.

To sum up: this is a carefully written, suitably illustrated textbook for technical assistants, the histopathological part of which, however, is so short that it hardly demonstrates the significance of histological phenomena related to various diseases. The detailed register and the alphabetical subject index make it easy to find one's way in the book.

SZ. GOMBA

Physiology of Movements. Encyclopedia of Plant Physiology New Series, Volume 7 by W. Haupt and M. E. Feinleib Springer-Verlag Berlin, Heidelberg, New York, 1979. 731 pp.

This volume provides an extensive survey of current research in those fields of plant movement in which essential progress has been made or important new aspects emerged in the past ten years. Twenty-seven leading scientists have contributed to the compilation of this work and they present the latest results from their own laboratories as well as a critical evaluation of the literature.

The book is divided into five main sections. The first part is a general review of plant movements and includes four chapters: "Introduction", "Stimulus Perception", "Reception and Transduction of Electrical and Mechanical Stimuli" and "Endogenous Rhythms in the Movement of Plants". The second part describes intracellular movements in five chapters: "Role of Microtubules in Intracellular Movements", "Actomyosin as a Basic Mechanism of Movement in Animals and Plants", "Cytoplasmic Streaming in Physarum", "Cytoplasmic Streaming and Cyclosis of Chloroplasts" and "Chloroplasts and Nuclear Migration". The third part deals with the locomotion of plants and contains two main microbial chapters: "Mechanism of Locomotion", "Control of Locomotion" and has seven subdivisions. The fourth part summarizes movements using turgor mechanisms and contains two chapters: "Movements of Stomata" and "Leaf Movements and Tendril Curling". The fifth part includes a discussion of growth movements and consists of the following main chapters: "Growth Movements Directed by Gravity", "Growth Movements Not Directed Primarily by External Stimuli".

There are many references to the relevant literature and a number of tables, illustrations and figures. The thorough and comprehensive material presented in this book provides a unique coverage of the biology and biochemistry of plant movements.

This volume will certainly be an outstanding guide to anyone interested in plant movements, in particular to biologists, biochemists and plant physiologists.

L. BOROSS

Neurozytochemie/Farbstoffe und Färbungen in der Histochemie by Ch. Pilgrim (ed.). Acta Histochemica 24. VEB Gustav Fischer Verlag, Jena 1981

This supplement volume of the Acta Histochemica contains the Proceedings of the 21st Symposium of the Histochemical Society (Gesellschaft für Histochemie) held in Gargellen, Montafon, Austria. The program of the Symposium was organized in three Workshops followed by free communications. Workshop 1 dealt with the normal and pathological nervous tissue. The acid phosphatase content in the developing chick spinal cord was determined with ultramicrochemical methods (van Welsum et al.). The combination of sophisticated cytological and histological techniques permitted the demonstration of various aspects of intraneuronal transport (Kreutzberg and Schubert). The intimate relationship between axonal smooth endoplasmic reticulum and the inner elements of the Golgi apparatus was described (Quatacker and DePotter). The neurotoxic effects of chemotherapeuticum Chlorochin was shown (Klinghardt). Workshop 2 covered the immunocytochemical demonstration of neuropeptides and neurotransmitters. The introductory review of van Leeuwen well summarized the theoretical and practical problems of this rapidly developing technique. It was followed by the detailed descriptions of various peptide and monoamine containing neuron systems in different animals. Sofroniew et al. studied the vasopressin, oxytocin and neurophysin systems in the hypothalamus and extrahypothalamic regions of human and primates. Pickel et al. described the enkephalin and tyrosine hydroxylase specific neurons in the neostriatum of fetal and adult rat. Monoamine containing neurons were characterized in the rat and lamprey (Steinbusch et al.). The SIF cells in the superior cervical ganglion was studied by immunocytochemical and biochemical techniques (Heym et al.). Lectures delivered on Workshop 3 (Dyes and staining techniques in the histochemistry) form the most voluminous and undoubtedly the most interesting section of the volume. Especially the first two speakers' reviews about biological stain (Wittekind) and about the basic principles of dye-substrate interactions (Zanker) call for wide interest. The subsequent papers dealt with various aspects of histochemical reactions including fluorescent dyes (Böhm and Fukuda), thiazidin dyes (Makovitzky), tetrazolium salts (Altman; Noorden et al.), Sudan Black B (Bayliss High; Frank et al. and Frederiks et al.). Horobin's contribution about the structure-staining relationship in biological staining gave a short review about the theoretical aspects of lipid staining. Among the free communications the authors reported various results obtained by immunohistochemical techniques and gave short accounts about technical and interpretational problems in histochemistry and autoradiography.

N. RÉTHELYI

Immunzytochemie by Ch. Pilgrim (ed.). Acta Histochemica 25. VEB Gustav Fischer Verlag Jena 1982

In this Supplementum of Acta Histochemica, the papers presented at the 22nd. Symposium of the Histochemical Society held in Würzburg with international participants from the FRG, Switzerland and Spain are collected.

Two major lectures were devoted to immunocytochemistry, the main subject of the Symposium. The first surveyed the theory and application of the methodology, and gave the critic of the different techniques. The second lecture focused on the generation and purification of antibodies using the actin-myosin system as a model of the problem.

A brief report gave account of the short papers, poster and slide presentations of a Workshop dealing with the advantages and disadvantages of the different immunohistological methods, and with the tests obligatory to prove antibody-, and method-specificity. In a model study measuring the concentration of aminopeptides in bile canaliculi, it was demonstrated that the PAP technique is 3 to 10 times more efficient, and 3 times more

sensitive than the immunofluorescence technique. Twelve additional short papers demonstrated practical application of the immunohistological methods. Detection of peptidergic and catecholaminergic neurons in primary cultures of the hypothalamus of the rat, demonstration of different enzymes, glycogen, amyloid, alfa-fetoprotein, tumor cell markers and viruses in experimental as well as in clinical material, clearly proved the usefulness of immunohistochemistry both in research and human pathology.

Ten papers under the title: "Free communications", completed the volume. They are common in applying histochemical methods to solve research or diagnostic problems, but are not related to immunohistochemistry. Among them the histochemical detection of lipids using OsO_4 staining, staining of chromatin with oxidized p-phenylenediamine or Alciane blue, new approaches to locate proteases, cellular localization of the uptake of 2 deoxy-D-glucose in brain tissue, and a study on spermiogenesis are interesting reports.

To sum up, this supplementum of Acta histochemica is a valuable collection of papers related to histochemistry.

G. SÉTÁLÓ

Quantitative Mikroskopie by H. Krug and R. S. Fritsch (ed.). Acta Histochemica 26. VEB Gustav Fischer Verlag, Jena 1982

This is a supplemental edition of 76 papers presented at the 11th Meeting of The Topochemistry and Electron Microscopy Society of German Democratic Republic (GDR) held in Leipzig in October 23–25, 1979. The overall evaluation of the presentations is given in the introductory remark of the editors. "Quantitative microscopy" is a common name for procedures in which the microscope is built into an instrument-complex suitable for quantitative measurements of cellular components visualized in the microscope. This field of the biological sciences and medicine has gone through a rapid development in the last two decades. Recent progress in quantitative microscopy is primarily due to the rapid development of new techniques especially modern electronics. Considering the techniques already applied in the field of the quantitative microscopy and those which might be applied in the future, it can be predicted that quantitative microscopy is only at the beginning of its development.

It was a right decision of VEB Gustav Fischer Co. to publish the papers presented at the 11th Meeting of the Topochemistry and Electron Microscopy Society of GDR as a Supplement. Such a summary has been due for a long time, since the last similar book was published in 1965. The individual techniques are not described in detail, instead the short papers presented and written by experts of the different research fields made it possible to summarize the status of quantitative microscopy in 1979.

The major headlines are the following:

1. Cytophotometry. The absorption methods (9 papers)
2. Cytophotometry combined with other methods. Interference microscopy (12 papers)
3. Cytophotometry combined with enzyme reactions (5 papers)
4. Fluorescence cytophotometry (13 papers)
5. Quantitative autoradiography (7 papers)
6. Polarization microscopy and other physical techniques (3 papers)
7. Morphometry (17 papers)
8. Different applications of morphometry (10 papers)

Under the headline Cytophotometry, 7 papers out of 9 are dealing with the measurements of DNA content of different cell nuclei after the Feulgen reaction. For cell biologists, the 2nd section is probably the most interesting part of the Supplement. Here the cytophotometric technique is combined with other techniques preferentially with interference microscopy. Most of the data presented in this 2nd section are giving new information about the quantitative aspects of cellular components mainly about the cell nucleus. In the third section, most of the papers are dealing with the tetrasolium salt indicators which complete the different enzyme reactions. Furthermore, there is also a good summary of the instrumental requirements for the quantitative estimation of the enzyme reactions. A broad review of the quantitative fluorescence microscopy can be found in the 4th section of the Supplement. In quite a number of papers, the applications of the fluorescence probes in nucleic acid and chromatin research are presented. The other

major topic is the quantitative immunofluorescence microscopy. There are also papers concerning flow cytofluorometry. Quantitative autoradiography is also dealt with. Beyond the estimation of the labelling index, the epidensitometric measurements of micro autoradiographs using image analyzers were also carried out. Only a small number of the publications deal with the polarization microscopic technique. The 7th and 8th sections are summarizing the relatively youngest field of quantitative microscopy, i.e. the morphometry and stereology of light and electron microscopic pictures. A large number of different techniques (area and volume measurements, image processing, automatic microscopical picture analysis etc.) have been applied here.

This Supplement will be a very valuabe and useful addition to the libraries of Cell Biology, Anatomy, Histology and Pathology Departments.

N. KELLERMAYER

Erzeugung, Messung und Anwendung ultravioletter Strahlen by T. Pfeifer (ed.). Fortschritte der experimentellen und theoretischen Biophysik 25. VEB Georg Thieme Verlag, Leipzig, 1980, 271 pp.

The book was published as the 25th volume of the excellent series, the Fortschritte der experimentellen und theoretischen Biophysik, (Progress in theoretical and experimental biophysics, editor Prof. W. Beier). Its editor was Th. Pfeifer (Physicist; VEB Quarzlampen, Markkleeberg) and it was written by an interdisciplinary authors' collective which consists of the most prominent specialists of the GDR working in this field. The book has a size of 270 pages, it contains 120 figures and 43 tables.

The book gives a wide-ranging survey of the results obtained up to now in the practical fields related to the application of UV light, among which the chemical industrial (the utilization of photochemical reactions), agricultural (the keeping of livestock, microflora) and further on the medical application are outstanding. Beyond this practical aspects the book discusses in a compact but high level form the theoretical bases of the physics of the UV light those of its generation and biological mechanism of action as well.

According to the above outlined problems the book is divided into three big chapters: the first one is "The physics of the UV light." In this K. Günther (Academy of Sciences of the GDR, Zentralinstitut für Elektronenphysik, Berlin) discuss the generation of UV light, its interaction with matter, besides this gives the theoretical bases necessary to understand the function of the devices to be introduced later on.

The second big chapter is "The practice of UV light". It includes the works of several authors and deals in great detail with the practical problems of the production and measurement of UV light. J. Moritz and J. Fischer (Glühlampenwerk, Berlin) review all the possible UV sources. The chapter gives all the data starting from natural UV radiation to UV lamps for special purposes and irradiating devices. A specially great number of tables and figures demonstrating well the main points are to be found in this chapter. — The author of the second part of this chapter is W. Falta (VEB C. Zeiss, Jena). He characterizes in detail the properties of optical materials used in the UV range. Based obviously on his own experiences he describes the main data and application of the optical elements and optical systems in the UV apparatures. — Also in the second chapter discusses H. Riegler (VEB C. Zeiss, Jena) the practical methods of the detection of UV light, the used circuits, types of detectors, multichannel optical analyzator systems. — The theoretical problems of the measuring technique are dealt with by J. Kaufhold (ASMW Fachabteilung Optik, Berlin), with special emphasis on the problems of absolute and relative measurements.

The third big chapter deals with the applications of UV light. It includes specially great amount of material, covering the most different fields of the application of UV light from livestock breeding to high precision measuring technique. H. Lang (ZIMET, Jena) treats the photochemical and photobiological effects of UV light in molecular and cellular level. This chapter emphasizes the role of UV light in the formation of life and UV light as an environmental damage as special problems. — G. Mehlhorn (Karl-Marx-Univ., Leipzig) studies the effect of UV light from agricultural aspects, from that of livestock

introducing UV light as a natural stimulus and a damaging physical phenomenon as well. Further on gives a short review on the possible practical utilization of UV light in livestock breeding (e.g. to increase the weight of calves and the milk production of cows, in pig breeding). — J. Barth (Karl-Marx-Univ., Leipzig) discusses in detail the effects of UV light on the human organism, the formation of erythema and pigment as the biological consequences of photochemical reactions. He deals also in detail with the photosensitizing properties of some pharmaceuticals and with the problems of photo-dermatoses. The reader gets a short survey on the diagnostical use of UV light on UV therapy and on some problems of photo-chemotherapy. — H. Voss (Ingenieur Hochschule, Köthen) discusses the application of UV light in chemical technology. He gives the theoretical bases of photochemistry and presents photochemical reactions which are generally used in industry. — A separate section of this chapter deals with the storage of the sun energy and with the theoretical problems of UV photoreactors and their practical realization. — S. Dähne (Academy of Sciences of the GDR, Zentralinstitut für Optik und Spektroskopie, Berlin) deals with the analytical use of UV light presenting shortly atomic absorption, absorption of luminescence spectroscopy. — At the end of the book W. Falta (VEB C. Zeiss, Jena) summarizes the measuring techniques (emission, absorption, luminescence, etc. spectroscopy, opacity, light scattering, determination of refractive index, etc) in which UV light plays a role.

From the above review it is obvious that the book is not written to people working in one single field but may prove useful for workers in several ones; it does not hold in sight the separate disciplines but it is problem centric: in its treatment UV light forms the guiding thread. Its great advantage that it surveys the problems of every field in contact with UV technique, giving thus high level up to date information for researches dealing with the production, measuring, application of UV light, with theoretical or practical problems (physicist, chemist, engineer, physician, veterinarian etc.). A further study of the details is helped by the abundant references at the subchapters.

G. Rontó

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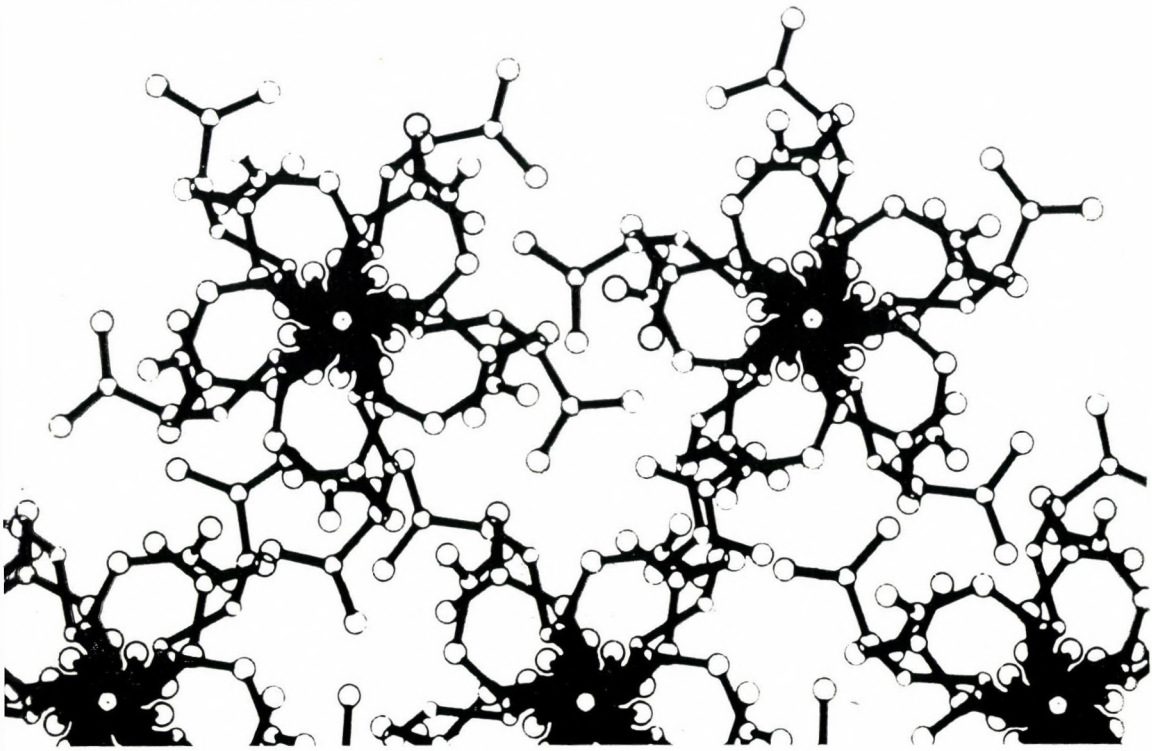

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