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Protein phosphorylation and dephosphorylation is a principal regulatory mechanism in the control of almost all cellular functions. The molecular properties and control of protein phosphatases that participate in these reactions have been the subjects of controversy. The interaction of liver phosphorylase kinase and phosphatase has been studied using different purification procedures. Rabbit liver extracts were further fractionated by gel filtration (Sepharose CL 4B), affinity chromatography (Heparin-Sepharose) or the combination of isotonic-hypotonic extraction.

Both procedures resulted in a fraction of high molecular weight (M_r 1.5 million D) enriched in kinase and phosphatase. The specific activity of phosphatase was increased 10-20 fold upon treatment with trypsin or freeze-thawing in the presence of mercaptoethanol. These treatments reduced the M_r of phosphatase dramatically (to 35,000 D). Phosphatase activity was inhibited by heat-stable inhibitors (1 and 2) and heparin in the low mol. weight form only. The activity of high mol. weight form was enhanced up to 4 fold by addition of polybrene (polycationic heparin antagonist) or histone fractions. Their effect could be due to the liberation of inhibitory proteins from the complex.

The strong interaction between phosphorylase kinase and phosphatase also supports the role of this kinase in the control of phosphatase activity. Preliminary data show a reversible inhibition of phosphatase activity upon addition of ATP-Mg. This inhibition could be attributed to the formation of the phosphorylated form of kinase.

HORMONAL REGULATION OF PHOSPHORYLASE PHOSPHATASE ACTIVITY IN RAT LIVER

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The activity of protein phosphatases involved in the regulation of glycogen metabolism is greatly influenced by the procedure of preparation. A new method has been developed for the assay of phosphorylase phosphatase activity and to detect its changes on hormonal treatment in crude liver extracts.

Phosphorylase phosphatase activity has been found to decrease by 50% in 3-10 minutes following the administration of glucagon or epinephrine and to return to the original value 15-20 minutes later. The phosphatase activity did not decrease on the effect of insulin but it increased by 30-40%. This reversible inhibition or activation of phosphatase was also observed in gel filtered extracts. It is concluded that hormones induce a change in the state of phosphorylation of proteins influencing phosphatase activity (phosphorylase kinase and heat-stable inhibitor-1) which may be important in the regulation of phosphatase activity in vivo.

The intravenous administration of fructose also resulted in a significant (50-60%) decrease of the phosphatase activity, but the inhibition was not observed in diluted extracts or Sephadex filtrates. Thus the phosphorylated proteins are not responsible for the inhibition of phosphatase, rather it is due to fructose-1-P accumulated in liver.

EFFECT OF FRUCTOSE ADMINISTRATION ON THE DEPHOSPHORYLATION OF PHOSPHORYLASE AND GLYCOGEN SYNTHASE IN RAT LIVER

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The dephosphorylation of phosphorylase and glycogen synthase has been studied in rat liver extracts and Sephadex G-25 filtrates. A latency was observed in the dephosphorylation of synthase as it became dephosphorylated (enzymatically active) only after the dephosphorylation of phosphorylase. In spite of the fact that the dephosphorylation of phosphorylase did not take place in liver extracts of rats treated with fructose, glycogen synthase became active. The latency reappeared in the dephosphorylation of synthase when the extract was filtered through a Sephadex G-25 column. The lack of latency is attributed to fructose-1-P formed following the administration of fructose in the liver. Fructose-1-P inhibits the dephosphorylation of phosphorylase a (and also its enzymatic activity) but does not influence the dephosphorylation of synthase. Fructose-1-P, added to the extract in vitro, suppressed the lag period caused by phosphorylase a without any effect on the dephosphorylation (and even on the catalytic activity) of synthase.

It is suggested that fructose-1-P accumulated following fructose administration induces conformational changes in phosphorylase a inhibiting the dephosphorylation reaction, and this modified enzyme cannot cause a latency in the dephosphorylation of synthase. Thus the active synthase converts glucose into glycogen resulting in hypoglycaemia following the fructose load.

SEPARATION OF PROTEIN PHOSPHATASES BY AFFINITY CHROMATOGRAPHY ON HEPARIN--SEPHAROSE

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Two classes of protein phophatases can be distinguished by the use of phosphorylated (inhibitor-1) and nonphosphorylated (inhibitor-2) heat--stable proteins. It is supposed that the inhibitory effect of these proteins is due to a peptide region with negative charges.

According to our results heparin inhibits the activity of phosphatases isolated from skeletal muscle or liver. The inhibitory effect of heparin and inhibitor-1 or 2 is additive. Polybrene, a heparin antagonist, relieves the inhibition of phosphatase caused by heparin or protein inhibitors. Two types of phosphatase are separated by chromatography on Heparin-Sepharose CL 6B using partially purified phosphatase preparation (M_r 33-35 kD). The fraction not bound to the matrix has phosphorylase and alkaline phosphatase activities and these activities are not inhibited by heparin or inhibitor-1 and 2. There is a twofold increase in the presence of 1 mM Mn^{2+}. The phosphatase bound to the matrix and eluted by 0.2 M NaCl has no alkaline phosphatase activity. Phosphorylase phosphatase activity of this fraction is inhibited by heparin, heat-stable inhibitors and 1 mM Mn^{2+}.

The chromatography on Heparin-Sepharose is also suitable for the separation of high molecular weight phosphatases.

REVERSIBLE PHOSPHORYLATION-DEPHOSPHORYLATION OF PHOSPHORYLASE IN DROSOPHILA MELANOGASTER

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Glycogen, the universal energy reservoir of animals, is mobilized by phosphorylase. Nonphosphorylated phosphorylase b was purified from D. melanogaster to homogeneity. Its kinetic properties and the assumed metabolite concentrations in the insect suggest that phosphorylase b is not active under physiological conditions.

The purified fruit fly phosphorylase b was interconverted into active phosphorylase a with the aid of ATP-Mg and phosphorylase kinase isolated from rabbit skeletal muscle. Rabbit skeletal muscle protein phosphatase reverted the above reaction.

The presence of phosphorylase kinase and phosphorylase phosphatase in D. melanogaster extract was demonstrated using rabbit skeletal muscle phosphorylase as exogenous, or fruit fly phosphorylase as endogenous substrates.

The activation of phosphorylase (b - a conversion) was correlated with the incorporation of radioactive phosphorus from (γ^{32}P) ATP into the enzyme. The phosphorylation of phosphorylase in vivo was also documented by 2D PAGE and autoradiography of the proteins obtained from the $^{32}P_i$ fed insects.

INTERACTION BETWEEN THE α- KETOGLUTARATE DEHYDROGENASE COMPLEX AND SUCCINATE THIOKINASE IN THE KREBS CYCLE

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Kinetic studies of the individual reactions of pig heart α-ketoglutarate dehydrogenase complex, succinate thiokinase and of the coupled system containing both α-ketoglutarate dehydrogenase complex and succinate thiokinase show that the K_m^{CoA} value of α-ketoglutarate dehydrogenase complex and $K_m^{succinylCoA}$ value of succinate thiokinase decrease in the coupled system as compared to those in the individual enzyme reactions. This phenomenon can be explained by the interaction between the α-ketoglutarate dehydrogenase complex and succinate thiokinase.

When they were centrifuged with 150,000 x g for 140 min, 25.4% of the succinate thiokinase sedimented in the presence of the α-ketoglutarate dehydrogenase complex, while no sedimentation was observed in the absence of the α-ketoglutarate dehydrogenase complex.

In gel chromatography experiments a significant peak of succinate thiokinase activity co-migrated with the α-ketoglutarate dehydrogenase complex peak.

By means of poly(ethylene glycol) precipitation, ultracentrifugation and gel chromatography we were able to detect a physical interaction between the α-ketoglutarate dehydrogenase complex and succinate thiokinase. Of the seven investigated proteins only succinate thiokinase showed association with α-ketoglutarate dehydrogenase complex, indicating the specific character of the association.

INVESTIGATION OF THE ACTIVE CENTRE OF EMULSIN β-D-GLUCOSIDASE

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The structure of the active centre of emulsin β-D-glucosidase (β-D-glucoside glucohydrolase EC 3.2.1.21.) has been studied by selective modification of the β-D-galactoside binding site in the presence of phenyl-1-thio-β-D-glucopyranoside.

The modification of the tyrosine groups located in the binding site was performed with N-acetyl-imidazole, a specific reagent.

The changes in activity, binding affinity and kinetic parameters (K_M, V) as a result of acetylation of the phenolic hydroxyl groups have been determined. The acetylation increased the K_M value in the case of β-D-galactosidase activity only, from 80 mM to 150 mM whereas that of β-D-glucosidase activity remained unchanged: 8.9 mM. Similarly the binding affinity for immobilized phenyl-β-D-galactopyranoside decreased to 66% and that for immobilized phenyl-β-D-glucopyranoside was unchanged: 98%.

The V value remained unchanged for β-D-galactoside hydrolysis (38 nmol/min) while it was decreased in the case of β-D-glucoside hydrolysis from 40 nmol/min to 20 nmol/min.

The non-competitive inhibition of the hydrolysis of phenyl-4-deoxy--β-D-glucopyranoside substrate by both glucono- and galactono-1,5-lactone showed that the C-4 hydroxyl group is essential in the appropriate binding.

Our conclusion is that there are two separate binding sites in the active centre of the enzyme; one is specific for β-D-glucoside, the other is specific for β-D-galactoside.

ISOLATION AND CHARACTERIZATION OF PANCREATIC ELASTOMUCOPROTEINASE

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Purified pancreatic elastase is accompanied by a minor component, elastomucoproteinase (EMPase), which can be separated and recovered in pure state by anion-exchange chromatography.

EMPase exhibits both proteolytic and mucolytic activities. Applying powdered aortic preparations as a substrate, the enzyme can split off carbohydrate moieties, but it can also cleave peptide bonds as a proteinase.

Assaying with tripeptide-p-nitroanilide substrates, the enzyme can decompose only those substrates which contain Phe or Tyr at the C-terminal. As regards binding Bz-Ala-Gly-Phe-pNA was the best peptide, while the hydrolysis of Bz-Ala-Pro-Tyr-pNA was the fastest.

Proteolytic activity of EMPase can be completely inhibited with phenylmethylsulphonyl fluoride, whereas its mucolytic activity only by 20%. The two activities may be located at two separate sites. The enzyme consists of a single polypeptide chain as judged by SDS gel electrophoresis in the presence of urea and β-mercaptoethanol. Its molecular weight is 25 000.

EFFECT OF EXPERIMENTAL CONDITIONS ON MYO-INOSITOL-1-PHOSPHATE SYNTHASE OF NEUROSPORA CRASSA STRAINS

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To investigate the thermostability of the enzyme, wild type and thermosensitive inl mutant microorganisms were cultivated at 22°C for 22 h, then at 37°C for 4, 8 and 24 h. It was found with the crude extracts that the amount and the activity of the enzyme was more significantly decreased at 37°C in the thermosensitive inl mutant than in the wild type cultures. When examining the thermostability (at various temperatures) of the enzyme in the crude extracts and that of the highly purified enzymes isolated from the same strains, there were differences in their degradation and their changes in activity upon heat-treatment. The enzyme obtained from the thermosensitive inl mutant exhibited stronger thermosensitivity.

During further experiments it was observed that protoplasts of the inl mutant strain 89601 are able to regenerate and grow on Vogel's minimal medium containing 1 M sorbitol. We assumed that the defective enzyme produced in this strain is activated upon the osmotic effect. Therefore the effect of sorbitol, L-sorbose and inositol on the purified enzymes isolated from various strains was studied. It was found that in the presence of sorbitol and L-sorbose the activity of the defective enzyme increased slightly. This may be due to the change in the steric structure of the enzyme. The hypertonic milieu had a strong inhibitory effect upon the enzyme isolated from the wild type and from the thermosensitive inl mutant.

Ca^{2+} SENSITIVITY OF MYOMETRIAL $NA^{+}K^{+}$ ATPase

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$Na^{+}K^{+}$ATPase activity in membrane preparations of various tissues can be abolished by millimolar levels of Ca^{2+}. However, micromolar concentrations of Ca^{2+} inhibit the enzyme activity in different vascular smooth muscles. We studied the $Na^{+}K^{+}$ATPase, and its Ca^{2+} sensitivity, of rat myometrium. Our results showed that $Na^{+}K^{+}$ATPase of rat myometrium can be assayed only after proper treatment with detergent. Na^{+} and K^{+} ions activated Mg^{2+} dependent ATP hydrolysis after preincubation with SDS. $Na^{+}K^{+}$ATPase activity was inhibited by 25-60 per cent by 10-100 μM Ca^{2+}. Sensitivity of the enzyme to Ca^{2+} was lost when proteins solubilized with SDS were separated from the membranous fraction containing the $Na^{+}K^{+}$ATPase activity by ultracentrifugation. Ca^{2+} sensitivity was restored by readdition of the solubilized proteins. The dialyzed supernatant was effective as well. $Na^{+}K^{+}$ATPase activity could be studied under better conditions after treatment of the microsomal fraction with dimethyl--sulfoxide and SDS: ouabain sensitive activation by monovalent cations was twofold increased, Mg^{2+}ATPase activity was tenfold decreased. Ca^{2+} sensitivity of $Na^{+}K^{+}$ATPase of the microsomal fraction incubated with DMSO before SDS treatment could not be eliminated by removal of the solubilized proteins.

Our interpretation is that a factor solubilized from plasma membrane by detergent is responsible for losing Ca^{2+} sensitivity of the $Na^{+}K^{+}$ATPase, and DMSO prevents solubilization of this membrane component. Inhibition of the $Na^{+}K^{+}$ATPase by low concentrations of Ca^{2+} may play a role in the regulation of the enzyme when adjusting to physiological changes.

FORMATION OF INTRAMOLECULAR DISULFIDE IN ADENINE NUCLEOTIDE TRANSLOCASE BY COPPER-O-PHENANTHROLINE

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A computer model has been proposed to describe the arrangement of the mitochondrial adenine nucleotide translocase in the membrane, on the basis of the distribution of hydrophobic and hydrophylic regions as well as homologous sequences of the molecule (M. Saraste, J.E. Walker, FEBS Letters 144 250-254, 1982).

The formation of an intramolecular disulfide by copper-o--phenanthroline in the adenine nucleotide translocase was demonstrated in the native state of the molecule, in submitochondrial particles and in isolated F_oF_1-complex. The proposed model would allow the formation of a disulfide between two particular cysteins of the four found in the polipeptide chain. It seemed worthwile examining whether the hypothetical and experimental results correspond to each other.

Peptide fragmentation procedures were used to identify the positions of the two cysteins forming the disulfide. Our results support the theoretical model.

It has been suggested that SH-disulfide interchanges may play a role in the function of the adenine nucleotide translocase (G.T. Robillard, W.N. Konings, Eur. J. Biochem. 127 597-604, 1982).

Our experimental observations on the internal disulfide formation in the adenine nucleotide translocase have been the first data supporting the hypothesis of the possible role of SH-disulfide interchanges in the function of the translocase.

USE OF CHROMOPHORIC LABELLING TO DETECT ANTIGEN INDUCED EFFECTS IN THE HINGE REGION OF RABBIT IgG ANTIBODY

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The molecular mechanism by which the initial antigen recognition on Fab is communicated to the Fc part to trigger effector mechanisms is still not clear. Signal transfer between Fab and Fc regions is expected to pass through the hinge region. The single interheavy chain disulfide occupies central structural position in the hinge of rabbit IgG, providing a suitable site for conformational probes. We used a covalent chromophoric label - 2,6-dimercuri-4-nitrophenol (DMNP) - inserted into this disulfide, to investigate the intramolecular propagation of the antigen induced signal. - Antiovalbumin IgG was raised in rabbits, and purified by affinity chromatography. Selective reduction of the interheavy chain disulfide was achieved by using mercapto-ethylamine under nitrogen atmosphere. The amount of "half molecules" (covalent H-L chain pairs) was more than 90 per cent in the preparations. The ratio of bound DMNP in the crosslinked half molecules was approximately 1 mole pro mole IgG as determined by spectrophotometric measurements. The inserted probe has a pK_a of 7.8, in comparison with a pK_a of 6.6 of the free label. This pK_a change and the observed red shift in the absorption maximum of the probe indicate a hydrophobic environment experienced by the probe. Upon formation of a soluble immune complex between labelled IgG and ovalbumin, a shift of the pK_a of the phenolic OH to 6.1 was observed. This change clearly shows that the signal is transmitted to the hinge region, and can be interpreted in terms of decreased hydrophobicity of the environment of the disulfide or by the appearance of negatively charged groups in its proximity. To estimate the intensity of the signal we calculated the free energy changes associated with the ligand induced changes in the interactions with the probe, which was -9.8 kJ $mole^{-1}$.

COOPERATIVE UNITS IN CONFORMATIONAL FLUCTUATIONS OF PROTEINS

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In native proteins buried, labile protons undergo isotope exchange with solvent hydrogens. The observed rate of H-D exchange is a sum of the rates of exchange from the folded state and that of the exchange mediated by reversible cooperative unfolding. Far under the melting temperature the exchange from folded state dominates, while approaching melting exchange occurs mainly by cooperative unfolding.

We suggest a simple method to estimate the size of cooperative units involved in H-D exchange near the melting temperature. We have found that the difference between the temperature where the exchange mediated by cooperative unfolding becomes dominating and the melting temperature of the protein structure, ΔT, is proportional to $M^{-1/2}$, where M is the mass of the cooperative unit in the folded ⇌ unfolded step. Therefore, this ΔT value may provide information on the size of cooperative units involved in large scale fluctuations of proteins and information on interactions of structural domains identified by X-ray crystallography.

This method has been applied for the human IgG molecule and its Fab and Fc fragments. The H-D exchange was monitored by infrared spectroscopy while the temperature of the protein sample was continuously increased. We suggest that from these complex exchange kinetics the transition temperature between the two exchange mechanism can be estimated.

IDENTIFICATION, ISOLATION AND LOCALIZATION OF VINCULIN IN BLOOD PLATELETS

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Vinculin, a recently discovered actin binding contractile protein that probably plays an important role in the attachment of microfilaments to the membrane in muscle and several nonmuscle cells was identified in platelets by competitive ELISA technique using antibodies raised against chicken gizzard vinculin. By modifying the method of Feramisco and Burridge (J. Biol. Chem. 255 1194, 1980) we succeeded in isolating bovine platelet vinculin. The structural identity of highly purified platelet and chicken gizzard vinculin was demonstrated by circular dichroism analysis. It was also shown that platelet vinculin binds to actin and induces a significant decrease in the viscosity of the F-actin solution. Vinculin was found to be localized in the submembraneous region and in the α granules by immunoelectronmicroscopic techniques and it was shown to be a substrate for activated plasma as well as platelet Factor XIII. It is presumed that vinculin is involved in the organization of actin filaments formed during platelet activation, especially in the linkage of newly formed microfilaments to the membrane.

P33: A NEW BASIC PLATELET PROTEIN

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Several proteins of highly basic character (platelet factor 4, platelet derived growth factor, permeability factors) with specific functions have been identified in blood platelets. Here we report the isolation of a so far unidentified basic platelet protein of 33 kD molecular weight termed as P33. Bovine platelets prepared from citrated blood were extracted at pH 3. The extract was submitted to ammonium sulfate fractionation, then it was applied onto a DEAE cellulose column in a medium containing 8 M urea. Fractions not bound to it were further purified by CM cellulose ion exchange chromatography. The single major protein peak eluted by the NaCl gradient contained a protein of 33 kD mole weight in an electrophoretically homogenous form. The amount of protein isolated was found to be around 0.05-0.1% of the total platelet protein. By isoelectric focusing and other electrophoretic techniques it was shown to posses a highly basic character. Unlike some other basic platelet proteins it did not bind calmodulin and so far no biological function could be attributed to it.

LOCALIZATION OF THE ALKALI LIGHT CHAIN BINDING SITE IN MYOSIN SUBFRAGMENT-1

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The binding site for alkali light chain (ALC) in rabbit skeletal myosin was studied on chymotryptic subfragment-1 (S-1) and its tryptic derivatives by crosslinking with ethylene glycolyl bis(succinimidyl succinate). One and two dimensional $NaDodSO_4$ polyacrylamide gel electrophoresis shows that ALC attaches to the heavy chain (HC) in the region of the 20 kD fragment which has been located at the S-1/S-2 junction of myosin. Crosslinked products of the 20 kD and 50 kD fragments and of 20 kD, 50 kD and 25 kD fragments also appeared and there was a complex of ALC and the three HC fragments as well. Ca^{2+} or Mg^{2+} reduces the extent of crosslinking and affects the changes in ATPase activities accompanying the crosslinking reaction. Chemical modification of the reactive lysine and cysteines abolishes the effects of divalent cations. These results are consistent with recent proposal suggesting that a region of HC adjacent to S-1/S-2 junction contains the ALC binding site (Szentkiralyi, Biophys. J. 37 39a, 1982) and also indicate divalent cation binding to HC of S-1. Considering these observations, and the fact that the ALC can be crosslinked with other crosslinkers to the 25 kD N-terminal fragment of S-1 (Labbe et al, Biochem. Biophys. Res. Commun. 102 466-475, 1981), it seems likely that ALC may interact with the HC on an extensively large contact surface.

STUDY OF THE ADP.VANADATE COMPLEX FORMATION OF DIFFERENT MODIFIED MYOSINS

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One of the intermediers of the myosin ATP-ase reaction is the M^{xx}.ADP.P complex which is the rate limiting, long living intermedier in this enzymatic reaction. Stimulators of the myosin ATP-ase (actin or specific modifications) loosen this complex.

In our work the HMM.ADP.vanadate complex (a stable analog of the above mentioned intermedier) was investigated to identify the essential side chains which are involved in the stabilization of this complex.

The ADP.vanadate binding was studied after blocking the reactive lysyl and -SH groups by TNBS, IAA and NEM. The enzymic activity was measured, and the structure of these complexes were studied by means of limited tryptic digestion in the presence of nucleotide.

On the basis of proteolysis and stability measurements we can conclude, that the most important factor in the stabilization of the HMM.ADP.vanadate complex is the divalent cation Mg^{++}. After blocking the essential lysyl-NH_2 group by TNBS the protein.$Mg^{\cdot ADP}_{\cdot vanadate}$ complex can't be stabilized, probably because this lysyl group is involved in the binding in a direct or indirect manner.

Furtheremore, it is concluded, that the essential -SH_1 group does not play any role in the formation of the ADP.vanadate complex. On the other hand, there is an unidentified side chain which reacts with NEM, and blocks vanadate incorporation.

THERMAL STABILITY OF MYOSIN SUBFRAGMENT 1.ADP.VANADATE COMPLEX

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Setton and Muhlrad (J. Muscle Res. Cell. Mot. 3 466, 1982) have reported a striking correlation between the loss of enzymatic activity and the change in the proteolytic pattern of myosin subfragment 1 (S1) subjected to mild heat treatment (2 h, 35°C). Instead of the three fragments of the native S1 heavy chain (27, 50, 20 kD), only two fragments (27, 20 kD) appeared on SDS PAGE after limited tryptic digestion of thermally inactivated S1. Addition of ATP, ADP or pyrophosphate, in high molar excess, prevented both the inactivation of S1 and the increased proteolytic susceptibility of the 50 kD fragment.

To investigate the mechanism of the protective effect two different stable analogues of the S1.ATP complex (S1.ADP.vanadate and S1.ADP trapped by crosslinking the reactive thiols) were subjected to tryptic digestion after heat treatment. The digestion pattern of the heat treated S1.ADP.vanadate complex was identical to that of the native one, indicating that the saturation of a single high affinity nucleotide binding site completely protected the protein structure against thermal denaturation, and the saturation of the hypothetical low affinity nucleotide binding sites have no influence on the thermal stability of S1. After heat treatment the S1.ADP complex, stabilized by crosslinking the essential thiols, prooved to be less stable than S1.ADP.vanadate complex, probably because of sterical constraints imposed on by the crosslinking of the reactive thiols.

THE NADH-VANADATE-OXIDOREDUCTASE ACTIVITY OF THE SARCOPLASMIC RETICULUM MEMBRANE

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The 5^+ oxidation state vanadium, the vanadate has a marked inhibitory effect on several membrane cation-transport enzymes. Its in vivo regulatory role on the Na^+K^+ ATPase was challenged by the fact, that vanadium exists inside the cells in the 4^+ oxidation state, as vanadyl cation. The conversion of vanadate to vanadyl was related to the surface membrane located enzyme, NADH-vanadate-oxidoreductase.

The sarcoplasmic reticulum Ca^{2+}ATPase forms extensive two-dimensional crystals upon treatment with vanadate. The study of the enzyme crystal revealed several new features of the structure and function of the enzyme, although the details of the enzyme-vanadate interaction are still unknown. The stabilization of the E_2 conformation proceeds in the presence of 0.1 mM vanadate, while the quick, extensive crystallization requires 5.0 mM.

The present work's goal was to examine whether the sarcoplasmic reticulum membrane has any NADH-vanadate-oxidoreductase activity. The enzyme activity was measured by the NADH oxidation rate (at 340 nm), in the presence of 0.05-2.0 mM vanadate. The non-enzymatic NADH oxidation was subtracted.

The sarcoplasmic reticulum membrane seems to have NADH-vanadate oxidoreductase activity. The K_m of the enzyme corresponds to 1.28 mM vanadate. The maximal velocity of the enzyme reaction was in the range of 200 nmol NADH/mg protein/minute. Millimolar concentrations of Ca^{2+} and Mg^{2+} seem to inhibit the enzyme activity. Mathematical analysis of the data suggests a complex, mixed type inhibition.

TWO-DIMENSIONAL CRYSTALLIZATION OF THE SARCOPLASMIC RETICULUM Ca^{2+}ATPase ENZYME

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Extensive two-dimensional arrays develop on the surface of native sarcoplasmic reticulum vesicles upon treatment with 5.0 mM vanadate in Ca^{2+} free medium. The same crystalline structure, observed on the surface of vanadate treated purified Ca^{2+}ATPase vesicles, gave evidence for the oligomer nature of the sarcoplasmic reticulum Ca^{2+}ATPase. The vanadate as inorganic phosphate analogue stabilizes the low Ca^{2+} affinity E_2 conformation of the enzyme and promotes the enzyme interactions.

The Ca^{2+}ATPase crystal preferentially develops on 600-700 Å diameter membrane tubules. The crystal lattice parameters are a: 61.9 Å, b: 114.2 Å, γ: 74.4°. The structural unit is the enzyme-dimer which forms chains and covers the tubule surface in right handed helix.

1.8 μM Ca^{2+} blocks the crystal formation and destroys the preformed crystallinity, inducing the conformation shift to the E_1 conformation. 5.0 mM ATP blocks the crystallization by vanadate, but remains ineffective on preformed Ca^{2+}ATPase crystals.

Preimposed inside positive membrane potential speeds up the vanadate induced crystallization. Inside negative membrane potential destroys the preformed enzyme crystals within seconds. This results show a direct effect of membrane potential on the Ca^{2+}ATPase enzyme conformation.

Similar crystalline structure was obtained in sarcoplasmic reticulum vesicles of slow, fast and cardiac muscle.

LOCALIZATION OF NEW PROTEOLYTIC FRAGMENTS DERIVED FROM SH-MODIFIED MYOSIN ROD

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Limited tryptic digestion of myosin rod produces well defined fragments, the positions of which have been established within the rod structure (Lu, Proc. Natl. Acad. Sci. USA 77 2010, 1980; Nyitray et al J. Biol. Chem. 258 13213, 1983). It has been reported that aging of heavy meromyosin (HMM), by oxidation of one or more SH-groups, changes the tryptic susceptibility of the α-helical portion of HMM (Balint et al J. Biol. Chem. 250 6168, 1975). During this work we compared the proteolysis patterns of native and SH-modified rods. There are 5 SH/chain in rod, 3 in subfragment-2 (S2) and 2 in light meromyosin. Interchain S-S crosslinks are formed by employing $CuCl_2$ or 5,5'-dithiobis(2-nitrobenzoic acid). In the digest of the oxidized rod (chain mass of 115 kD) three new bands appear on $NaDodSO_4$-gels: chains of 100 kD, 45 kD, and 25 kD. Tryptic digestion of oxidized long-S2 (58 kD) or short-S2 (37 kD), isolated from the digest of reduced rod, produces the same 45 kD and 25 kD fragments, respectively. Partial sequence-analysis of the isolated new fragments (45 kD and 25 kD) indicates that they are formed by the removal of 97 residues from the N-terminus of rod long-S2. The new cleavage site is located in the vicinity of one of the modified SH-groups (Cys-105). These data show that SH-modification (especially oxidation) of myosin rod leads to a local disruption of the coiled-coil structure near the functionally important head/rod "swivel".

CHANGES OF LYSINE REACTIVITIES OF ACTIN IN COMPLEX WITH DNASE I

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In an attempt to determine which part of the actin molecule participates in its interaction with DNase I we compared the reactivities of lysine residues in G-actin to those in actin complexed to DNase. Limited reductive methylation was carried out on G-actin and the actin - DNase complex with (^{14}C)HCHO and (^{3}H)HCHO, respectively. (^{3}H)actin and (^{14}C)actin were combined and the lysine containing peptides were isolated. The ^{3}H/^{14}C ratio in a peptide containing the i-th lysine residue was taken as the reactivity of the i-th lysine in the complex relative to that in G-actin.

We found that the reactivities of Lys-61 and -68 decreased by about 50% while that of Lys-290 increased by about 50% upon the actin - DNase interaction. Since polymerization also reduces the reactivities of Lys-61 and -68 (R.C. Lu, L. Szilagyi, Biochemistry 20 5914-5919, 1981) these results suggest that DNase I inhibits the polymerization of actin by blocking one of the actin - actin contact regions. Our results are in good agreement with other reports confining the DNase binding site to the N-terminal third of actin (H.G. De Couet, J. Muscle Res. Cell Motility 4 405-429, 1983).

THE EFFECT OF INCREASED NEUROMUSCULAR ACTIVITY ON THE MUSCLE DEVELOPMENT IN CHICK EMBRYO

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Several investigations support the hypothesis of an influence of motor innervation upon the differentiation of muscle fiber types. To test this supposition we performed some enzyme assays - reflecting the state of muscle differentiation - of 3 different muscles in 4-aminopyridine (4-AP) treated and in control chick embryos. 4-AP selectively blocks membrane K^+ conductance and hence prolongs action potentials both in nerve and in muscle; it increases the number of transmitter packages released by each nerve impulse.

The eggs were injected 4 times with 0.1 mg 4-AP (or with NaCl of 0.9%) during the critical stage of muscle development. 2 fast-twitch muscles (breast muscle, PLD) and a slow-tonic muscle (ALD) were dissected from the embryos one day before hatching. The specific enzyme activities of CK, LDH and the ratio of isoenzymes (subunits) were determined from the water-soluble supernatants of muscle homogenates.

In ALD a substantial increase of CK-MB isoenzyme and of the H subunits of LDH enzyme was observed following the treatment (approx. +30% as compared to control values); the total activities of LDH and CK enzymes were reduced to a smaller extent. No great changes could be noted in the fast-twitch muscles of treated embryos.

Our results indicate that enhanced neuromuscular activity accelerates the development of the slow muscles, and thus it is plausible to suppose that nerve impulses are needed mainly for the differentiation of slow muscles.

THE PROPERTIES OF SARCOPLASMIC RETICULUM MEMBRANE (SR) FROM RABBIT M. GASTROCNEMIUS IN DIFFERENT PHYSIOLOGICAL CONDITIONS

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Restriction of the motility of muscles, for example the strainless immobilization of muscle by plaster cast, leads to atrophy. The membrane system of sarcoplasmic reticulum (SR) - one of the regulating factors of muscle contraction - shows considerable transformation in transforming muscle fibers. The changes seemed to be reversible. After removal of plaster cast from immobilized leg the atrophied muscle has gained weight and the recovery of characteristic parameters (Ca^{2+}-ATPase activity and Ca^{2+} accumulating capacity) has taken place. In parallel with the measurement of functional parameters the changes in the amount and quality of different lipid components (phospholipids, cholesterol, unsaturated fatty acid etc.) were followed. It seems obvious that the time period of immobilization has a definite influence on the quantity and composition of the phospholipids in the SR. According to the generally accepted view the function of SR in the requlation of contraction and relaxation of muscle is exerted through the release and accumulation of Ca^{2+}. The evidence accumulated over the last years indicated that the lipid environment (first of all the phospholipids) is essential for both the ATPase reaction and the coupled calcium transport in SR. The physical state of the phospholipids associated with the Ca^{2+}-ATPase enzyme is an important factor in determining the rate of ATP hydrolysis in SR. The effects of the temperature and solubilization on enzyme activity, lipid fluidity and mobility of protein have indicated a correlation among these variables. From our experiments it became quite obvious that the immobilization and the following regeneration of muscle make profound alterations in the lipids of SR. It seems reasonable to suppose that these changes affect the ATPase activity of SR and so its regulatory role. The demonstrated results point to a possible alteration in membrane fluidity. Further experiments are needed to elucidate the microenvironmental changes around ATPase oligomers.

CLONING AND CHARACTERIZATION OF 1.5 AND 2.5 KB EcoRI REPEATS OF NOVIKOFF HEPATOMA TUMOR CELL GENOM

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Long, repeated sequences of the Novikoff hepatoma tumor cell genome containing several thousand base pairs were studied. The sizes of the most frequently occurring repeats, obtained by EcoRI enzyme, were found to be 1.5, 2.5 and 6.0 kb.

Several independent clones, containing the 1.5 and 2.5 kb EcoRI repeats were isolated. Restriction mapping of these independent clones showed a minor heterogeneity. A detailed map of the whole long repeat in the genome was constructed, using one of the cloned fragments obtained by nick translation. The constant region of the 2.5 kb repeat has been sequenced.

TRANSFORMATION OF NEUROSPORA CRASSA WITH RECOMBINANT DNA

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A Neurospora (N.) crassa gene library was constructed inserting 30-40 kb MboI fragments of N. crassa DNA into BamHI site of cosmid BHB 3030. A cosmid pool isolated from the gene library was used to transform an inl (inositol-requiring) mutant of N. crassa. The inl$^+$ transformants obtained in this experiment were back-crossed with an inl strain and individual inl$^+$ ascospores of the progeny were selected for further study.

The transformed trait proved to be inherited as a chromosomal marker. Cross-immunoelectrophoresis of the gene product suggested that the transformants contained both the mutant and the intact gene copies.

In Southern-hybridization experiments bands of plasmid mobility were detected in the inl$^+$ clones. Eleven recombinant plasmids of different restriction patterns were recovered from these clones by means of E.coli transformation. These plasmids are under investigation for their transforming ability on inl mutant. Until now one of them proved to be positive in Neurospora transformation experiments.

The plasmids were maintained during the meiosis, therefore they are supposed to have replication origins. Such sequences might be useful for the construction of new vectors for N. crassa.

STRUCTURE OF THE MODIFICATION METHYLASE GENE OF THE PHAGE SPR

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Due to their well-defined and relatively simple action on DNA, type II restriction-modification systems are attractive subjects of studies on specific DNA-protein interaction. The genes of three different methylases of similar sequence-specifity were previously cloned in our laboratory. In order to understand the structural basis of the specificity we wanted to determine the nucleotide sequence of these genes. A 2.1×10^3 base-pair segment of SPR phage DNA cloned in Escherichia coli has previously been shown to carry the functional SPR methylase gene. The approximate location of the gene on this DNA segment was established, and the nucleotide sequence of the relevant region was determined by the Maxam-Gilbert procedure. An open reading frame that can code for a 439 amino acid protein was found. The calculated molecular weight (49822) of this protein is in fair agreement with previous estimates (49000). The synthesis of this protein was demonstrated in E.coli minicells. The initiation point of transcription was localized by S1 nuclease mapping. The deduced amino acid sequence of the protein shows significant homology with the previously determined Bacillus sphaericus R methylase sequence. A 114 amino acid long region of the two methylases shows 41% homology.

ANALYSIS OF A CIS-ACTING REGULATORY MUTATION IN DROSOPHILA MELANOGASTER

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A third chromosomal inversion was found to carry a large extra puff at 87B region in salivary gland, not existing in any other Dr. melanogaster stock. This puff has no effect on the other homologue, therefore must represent a cis-acting regulatory change in the affected region. We have shown this morphological phenomenon to be associated with excessive RNA and protein synthesis. We are currently working on the cloning of this region by chromosomal walking method in order to determine the nature and extent of the mutational change.

MIDDLE REPETITIVE DNA SEQUENCES IN DROSOPHILA HYDEI

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To isolate Drosophila hydei specific middle repetitive sequences, we set up a pBR 322 library containing BamHI cleaved DNA segments and selected for positive colonies by hybridizing total nick-translated D. hydei DNA to "Grunstein-Hogness" filters. 52 clones were chosen for further analysis and hybridized to total genomic D. hydei DNA individually. Nearly half of the clones showed patterns of hybridization characteristic of dispersed repetitive DNA.

These clones were used for in situ hybridization to salivary gland chromosomes of D. hydei. Only two of the clones proved to be slightly dispersed repetitive (4-5 sites) and the majority hybridized mainly to the chromocenter and the nucleolus.

Our results so far confirm previous findings that much smaller numbers of dispersed repetitive elements can be detected, even in sibling species (D. simulans, D. mauritiana) than in D. melanogaster.

EFFECT OF GAMMA IRRADIATION ON THE PHOSPHORYLATION OF PLANT CHROMATIN PROTEINS

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Pea seeds were irradiated with 300 Gy dose of cobalt gamma irradiation, and germinated for 7 days in the dark. From the detached etiolated shoots, chromatin was isolated and phosphorylated by chromatin--bound protein kinases. Phosphorylation of chromosomal proteins was increased by 300 per cent in chromatin isolated from irradiated plants. Phosphorylated chromatin proteins were subsequently divided into three fractions: histones, 0.05 M TRIS (pH 7.5) soluble and non-histone proteins. These proteins were separated by polyacrylamide gel electrophoresis and their radioactivity was detected by autoradiography. It was found that the irradiation increased the overall phosphorylation more than three times in the histone fraction. The H4 histone was not phosphorylated. The phosphorylation of H1 histones was higher in the control, while the phosphorylation of H2B, H2A and H3 histone fractions was higher in the irradiated sample. Between the H1 and H2B histones a highly phosphorylated, new band appeared. There were no changes in the phosphorylation of 0.05 M TRIS (pH 7.5) soluble and non-histone fractions.

ISOLATION OF A MULTIENZYME COMPLEX OF REPLICATION FROM TONSILLAR LYMPHOCYTES

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It was reported earlier that in S phase fibroblasts (Reddy, Pardee, Nature 304 86, 1983) and in lymphoblasts (Wickremasinghe et al, FEBS Letters 159 175, 1983) the nucleotide synthesizing enzymes and the DNA polymerase-α were associated in a multienzyme complex. Our previous results suggested that in lymphocytes the utilization of nucleosides was directed by enzymes associated in a complex. The DNA synthesizing enzyme complex was prepared from unseparated lymphocytes and from separated, activated B lymphocytes. The cells were lysed by hypotonic treatment followed by phospholipase C and Micrococcal nuclease digestion. The cell lysate was chromatographed on a Sepharose 6B column. The molecular weights of the free enzymes were determined in the presence of 1M KCl. In this salt extract the molecular weights of the DNA polymerase-α, thymidine kinase and deoxycytidine kinase were found to be 100,000, 60,000 and 60,000, respectively. In the case of the crude lysate the molecular weights of kinases and DNA polymerase-α were shifted to above 10^5. The amount of the high-molecular weight component was higher in activated B lymphocytes than in unseparated cells.

The role of the replication complex in the channeling of precursors into DNA is the subject of further investigations.

THE INCORPORATION OF ^{3}H-THYMIDINE AND ^{3}H-DEOXYCYTIDINE BY PERMEABILIZED HUMAN LYMPHOCYTES

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Tonsillar lymphocytes were permeabilized by hypotonic treatment. Cells acquired the capacity of nucleotide-dependent incorporation of ^{3}H-TTP into DNA, while the ability to incorporate ^{3}H-thymidine (Thd) was lost and the incorporation of ^{3}H-deoxycytidine (dCyd) decreased to about 50% of that of the intact cells, suggesting some defects of the key enzymes involved in nucleoside salvage. In order to elucidate the fate of exogenous Thd and dCyd, the phosphorylation of both nucleosides was determined in permeabilized and in intact cells. Phosphorylated Thd accumulated in the form of dTMP only, while a considerable part of phosphorylated dCyd was found as dCDP and dCTP by PEI cellulose chromatography. The specific activities of deoxycytidine kinase, deoxythymidine kinase and DNA-polymerase (measured in the cell homogenate) were not changed during the short permeabilization procedure. These data suggest that the dCyd salvage pathway is less sensitive to hypotonic treatment, i.e. to the damage of the cell membrane, and dCyd is channeled into DNA by a way different from that of dThd.

CHARACTERIZATION OF THREE PLASMIDS OF AEROMONAS HYDROPHILA

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STEROID BINDING PROPERTIES OF THE PARTIALLY PURIFIED GLUCOCORTICOID RECEPTOR

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Chick thymus glucocorticoid receptor was partially purified by affinity chromatography. A novel affinity matrix was prepared in order to recover the purified receptor in a form still capable of binding steroid ligands. Sepharose 4B was modified to contain SH functions on a long arm and cortexolone-21-mesylate was coupled to this group through a thioether bond. This method of derivatization was significantly simpler and faster than those described in the literature. The gel bound the receptor protein with a relatively low affinity so that it could be recovered by the use of steroids that rapidly dissociate from the binding site. Steroid specificity of the partially purified, uncomplexed receptor could be studied in this way.

Purification was 10-20 fold when it was performed as a batch procedure and 100-150 fold when the affinity gel was used in a column. Kinetic studies done with the partially purified receptor showed that its steroid binding properties were basically similar to those of the crude receptor preparates of the cytosol.

ANGIOTENSIN II STIMULATES PHOSPHATIDYLINOSITOL TURNOVER IN ADRENAL GLOMERULOSA CELLS

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In the present experiments we compared the effects of agents stimulating aldosterone secretion on phosphatidylinositol metabolism. Glomerulosa cells, isolated from rat adrenals, were incubated in the presence of angiotensin II, elevated potassium concentration, corticotropin, dibutyryl cyclic AMP and prostaglandin E_2. Of all these substances, only angiotensin II stimulated the incorporation of ^{32}P--phosphate into phosphatidylinositol. In cells prelabelled with ^{32}P--phosphate, the radioactivity in phosphatidylinositol decreased in response to angiotensin II within 5 minutes. In further experiments, we examined whether this phenomenon requires the presence of extracellular Ca^{2+}, and its sensitivity to inhibition by cycloheximide. It was found that neither the stimulation of phosphatidylinositol breakdown nor the stimulation of incorporation of ^{32}P-phosphate into phosphatidic acid and phosphatidylinositol required the presence of extracellular Ca^{2+}. Cycloheximide abolished the effect of angiotensin II on aldosterone production, but not on phosphatidylinositol synthesis. The results suggest that angiotensin II increases the phosphatidylinositol turnover of adrenal glomerulosa cells. This effect may precede, but does not depend on, angiotensin-induced Ca influx.

EFFECT OF ANGIOTENSIN II ON THE TURNOVER OF POLYPHOSPHOINOSITIDES IN ADRENAL GLOMERULOSA CELLS

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Hormones and neurotransmitters which act by changing cytoplasmatic calcium concentration were found to alter the metabolism of phosphoinositides. It has been suggested that these alterations may have significance in the generation of the calcium signal. Evidence is accumulating on the importance of the phosphorylated metabolites of phosphatidylinositol (PI) - diphosphoinositide (DPI) and triphosphoinositide (TPI) - in this effect.

Previous work in our laboratory showed that angiotensin II stimulates the breakdown as well as the resynthesis of PI in isolated glomerulosa cells.

In the present experiments we examined the effect of angiotensin II on the a/ incorporation of ^{32}P phosphate into TPI in glomerulosa cells, b/ breakdown of DPI and TPI of glomerulosa cells preincubated in the presence of ^{32}P phosphate. Angiotensin stimulated the breakdown of DPI and TPI as well within 15 seconds. DPI and TPI content decreased by about 30-35%. Angiotensin II did not enhance the incorporation of ^{32}P phosphate into TPI during the first 30 minutes of stimulation. Considering that after the initial decrease DPI and TPI content remained on the same low level for at least 30 minutes, these results suggest that the increased breakdown of DPI and TPI lasts only for seconds.

The present data indicate that changes in phosphatidylinositol and polyphosphoinositide metabolism after stimulation of the cells with angiotensin II are essentially different.

APPARENT AFFINITY OF DIHYDROTESTOSTERONE, TESTOSTERONE, AND NORTESTOSTERONE TO THE ANDROGEN RECEPTOR UNDER VARIOUS CONDITIONS - - CORRELATION WITH THEIR EFFICACY IN VIVO

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The androgen receptor-binding properties of dihydrotestosterone (DHT), testosterone (T) and 19-nortestosterone (NT) were compared in cell-free extracts prepared from the seminal vesicles of castrated rats. Competition experiments showed that these steroids bind to the same receptor sites. Saturation analysis performed using two different sets of conditions (incubation time and temperature: 90 min, 0°C and 22 hrs, 15°C) indicated different orders of apparent affinities (NT>T>DHT and DHT>NT>T, resp.). The primary cause of this change in affinity order was a dramatic decrease of the binding affinities of NT and T. At 0°C NT bound to the receptor 2-3 fold more rapidly than either T or DHT (NT>T≈DHT). Dissociation rate constants showed the following order of stability of these steroid--receptor complexes: DHT>NT>T. Furthermore, from the kinetic constants a sequence of affinities could also be calculated (DHT≈NT>T). These results suggest that (a) using 0°C and short incubation time, the apparent affinity of a steroid to the receptor reflects predominantly the rate of association of the ligand, (b) affinity constants calculated from the kinetic constants or determined by saturation analysis under conditions that favor the attainment of binding equilibrium are more indicative of the in vivo efficacy of the steroids, (c) assessment of stability of the steroid-receptor complex seems particularly useful in predicting the in vivo effects of the steroid ligand.

EFFECT OF CHRONIC TREATMENT OF NORMAL ADULT RATS WITH ESTRADIOL AND PROGESTERONE ON THE RATE OF INCORPORATION OF 3-H ARACHIDONATE INTO VARIOUS LIPIDS AND PROSTANOIDS IN THE UTERUS

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Daily i.p. injections of 0.2 mg estradiol phenylpropionate (EPP) or 0.2 mg EPP + 2.0 mg progesterone (P) into adult female rats had only minor trophic effects on the uterus. When minced uteri were incubated with (^{3}H)arachidonate ((^{3}H)AA), it was found that EPP-treatment caused a 2-3-fold, selective increase over the control value in the rate of labeling of some of the phospholipids and the triacylglycerol fraction. These stimulating effects were either not influenced or were diminished by P, except phosphatidyl inositol, the labeling of which was increased to 200% of the control value after EPP treatment and to 300% after EPP + P treatment. - The biosynthesis of prostanoids from (^{3}H)AA was studied using microsomes prepared from the uteri of hormone-treated and control rats. After EPP-treatment the synthetic rates of $PGF_{2\alpha}$ and PGE_2 decreased dramatically but this decrease could partially be prevented by coadministration of P with EPP. The formation of 6-keto-$PGF_{1\alpha}$ was significantly inhibited by EPP treatment only when glutathione was added to the incubation mixtures. However, P had no effect in this case. These results indicate that the acylation-deacylation reactions of certain lipids and the biosynthesis of prostanoids from arachidonate are major targets for female sex steroids in the normal rat uterus. (Supported by MTA, Hungary and NSF, USA.)

MOULTING HORMONE PLAYS A ROLE DURING THE EMBRYOGENESIS OF DROSOPHILA MELANOGASTER

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Moulting hormone - mainly ecdysone - can be detected by radioimmunoassay in Drosophila embryos. The hormone level exhibites a maximum (240 ng/g protein) 8 hours after fertilisation. It seems that the hormone is formed via an apolar, rather than a highly polar precursor. Therefore the hormone is not liberated by a single enzymatic step from a polar precursor of maternal origin.

Moulting hormone receptors can be demonstrated in the embryos, too. The hormone binding studies, using either cytosol or homogenate of the eggs, indicate a single binding site with 1.6×10^{-9} M binding constant for ^{3}H-ponasterone A. The level of the binding moiety is negligible during the first half of embryogenesis, it rises after the hormone level maximum and peaks at 14 h, than declines rapidly, being negligible again when larvae heach.

The above findings suggest that the moulting hormone has some role during embryogenesis. Furthermore, it seems that there are two ways of the regulation of ecdysone dependent processes: the hormone level as well as the receptor level is changed at various developmental stages.

INVESTIGATION OF PROSTANOID METABOLISM IN MOUSE LIVER

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Prostanoid synthesis was investigated in perfused livers, in isolated hepatocytes and non-parenchymal cells and in their homogenates. Isolated hepatocytes were prepared by the collagenase perfusion method. Arachidonic acid metabolites - Thromboxane B_2 (TxB_2,/, 6-keto--prostaglandin $F_{1\alpha}$ (PGI_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) - were determined by radio-immuno-assay. TxB_2 and PGI_2 levels referred to fresh weight were increased in perfused livers and in the perfusion medium in the course of a 30 min incubation while $PGF_{2\alpha}$ was increased only in the livers but not in the medium. Prostanoid production could be inhibited by indomethacin. Isolated hepatocytes were shown to synthesize TxB_2, PGI_2 and $PGF_{2\alpha}$. In the homogenates of the hepatocytes a 3-5-fold level of these prostanoids was found. Isolated non-parenchymal liver cells did not synthesize prostanoids measurable by radio-immuno-assay. But when homogenates prepared from non-parenchymal cells were incubated with ^{3}H-arachidonic acid, labeled PGE_2 and $PGF_{2\alpha}$ but not TxB_2 were obtained. These results suggest that hepatocytes are responsible for the TxB_2 synthesis in the liver.

REGIONAL SPECIFICITIES OF PGE_2 BINDING AND PROSTANOID BIOSYNTHESIS IN THE SMOOTH MUSCLE LAYER OF TWO FUNCTIONALLY DIFFERENT PORTIONS OF THE HEN OVIDUCT

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Specific PGE_2 binding to microsomes prepared from the smooth muscle of the vaginal and uterine portions of the hen oviduct was characterized with respect to temperature dependence, time course of association and dissociation and concentrations of PGE_2 and membranes. It was found that the presence of 9-oxo, 11 and 15 -OH groups were essential for optimal binding. Saturation analysis (at 37°C) indicated a single class of high affinity binding sites in the vagina (K_d~1 nM), whereas the uterus exhibited two kinds of receptor populations (K_{d1}~1 nM, K_{d2}~20 nM). Binding in both portions occurred to a limited number of receptor sites (14, 19 and 116 fmole/mg protein). Vaginal microsomes produced about four times more prostanoids from (^3H)-arachidonate than did uterine microsomes. In the presence of adrenaline both tissues synthesized mainly TxB_2, PGE_2 and significantly less $PGF_{2\alpha}$. Addition of glutathion or cytosol markedly increased the yield of PGE_2 at the expense of TxB_2. However the PGE_2/TxB_2 ratio of synthetic rates was always about 2-fold higher in the vagina than in the uterus. Only trace amounts of PGI_2 (measured as 6-keto-$PGF_{1\alpha}$) and PGD_2 were produced. We suggest that PGE_2 produced in relatively high concentrations in the vagina exerts its known relaxant activity in the vagina and its contractile action in the uterus by acting via different receptor sites (Supported by MTA, Hungary, and NSF, USA).

PLANT PHYSIOLOGICAL EFFECTS OF $PGF_{2\alpha}$ AND PROSTAGLANDIN SYNTHESIS IN CORN-LEAF

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Recent investigations have given evidence that PGA_1 is found in onions, and $PGF_{2\alpha}$ and PGE_2 in red algae.

We have found that corn-leaf contains the enzymes which convert the arachidonic acid into prostaglandins. Corn-leaf homogenate was incubated with ^{3}H-labelled arachidonic acid. After a selective extraction procedure the reaction products were separated using thin layer chromatographic techniques. $PGF_{2\alpha}$ and PGE_2 were identified among the radioactive reaction products by rechromatography of the PG-derivatives formed from them.

Effect of $PGF_{2\alpha}$ on photosynthesis was tested in several plants. The rate of photosynthesis was reduced in every case. Corn-leaf segments were floated on $PGF_{2\alpha}$ solutions of different concentrations for a day. The solution of 200 ppm caused about 50% reduction in the incorporation of $^{14}CO_2$, ^{3}H-thymine and ^{14}C-glycine.

The results that corn-leafs contain cyclo-oxigenase activity, and $PGF_{2\alpha}$ has a significant effect on photosynthesis, amino acid incorporatio and nucleic acid metabolism, confirm the hypothesis that prostaglandins exist in plants and the role played by prostaglandins in plants is as important as that in animals.

CURRENT PROBLEMS OF INTERFERON AND LYMPHOKINE RESEARCH

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Interferons will be described briefly with regard to nomenclature, physicochemical properties, genes, protein structures and various biological effects. The mechanisms of action under in vitro and in vivo conditions and their influence on the immune system will also be summarized. Particular interest will be paid to the biotechnological aspects of the large-scale production of interferons. The regulation of interferon synthesis and the mechanism of priming will be cutlined. Current results of human clinical studies with natural and recombinant interferons in tumors and viral diseases will also be summarized.

Lymphokines, and particularly migration inhibiting factor, leukocyte inhibitory factor, macrophage activating factor, interleukin 1 and interleukin 2, will be described from the aspect of their heterogeneous activities and their possible role in host defence mechanisms. Evidence in support of connections and mutual regulation between interferons and lymphokines will be reviewed.

The present state of interferon and lymphokine research in Hungary and future prospects will be discussed.

ENTRAPMENT OF EUKARYOTIC CELLS FOR CONTINOUS CULTURE OPERATION

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Immobilization of enzymes and procaryotes is attracting great attention. Immobilization of microbial cells and organelles permits the immobilization of multienzyme and coopęrative enzyme systems. Immobilized cells were used for industrial production of several amino acids, vitamins and biotransformation of various compounds (steroids). The gel matrixes used are natural polymers (alginate, cellulose, gelatine, collagen, agar) or synthetic polymers (polyacrylamide, acrylates and various acrylate derivatives).

We devised a technique for the entrapment of macrophages and spleen cells in alginate beads. The alginate beads proved to be suitable for the continous production of various enzymes, immunoglobulins and lymphokines.

CLONING OF E.COLI rRNA PROMOTERS AND THEIR USE FOR THE EXPRESSION OF OTHER GENES

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The transcription on E.coli rRNA operons is the most intensive transcription on any bacterial gene and it is subject to several elaborate control mechanisms. In our earlier work, the detailed physical map of the 7 rRNA operons has been established and restriction endonuclease-generated fragments carrying total rRNA operons have been cloned with the plasmid vector pBR322.

Nucleotide sequence analysis and in vitro transcription experiments with the constructed recombinant plasmids revealed a complex promoter region in front of the rrnB operon. Recently we presented evidence for the existence of four promoters located about 200, 300, 1200 and 1300 bases upstream from the start of the mature rRNA sequence.

We have shown that cloning and stable maintenance of the very strong rRNA promoters on a multicopy plasmid is possible by deleting the structural part of the operon and by fusing the terminators of the gene immediately to the promoters. Several deletions were constructed in vitro in the rrnB operon, in order to vary the promoter-promoter, promoter--terminator distances or to destroy any of the promoters or terminators. The constructed recombinant plasmids provide a convenient experimental system to study the regulation of rRNA biosynthesis.

The possibility whether the strong rRNA promoters can contribute to the expression of genes coding for proteins and the construction of a plasmid vector in which the rRNA promoters direct foreign gene expression, will be discussed.

CONSTRUCTION OF SHUTTLE VECTORS FOR CLONING DNA IN STREPTOMYCETES

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In the past 40 years different strains of Streptomycetes were used for the production of antibiotics for the human therapy. Cloning and engineering of genes involved in the biosynthesis of antibiotics may make possible a higher yield and the construction of strains producing new structures. DNA cloning vectors carrying determinants for antibiotic resistancy and capable of replication in Streptomycetes and E.coli have been constructed. Essentially, plasmid pIJ41 isolated from S. lividans was cut with XhoI and ligated to pACYC 184 linearized with SalI. Transformants were selected by Tc sensitivity. The restriction map of pGYOKI 1 and pGYOKI 2 (pACYC 184 in both orientations) was constructed. In E.coli, the neomycin phosphotransferase gene was expressed followed by spontaneous interplasmidic recombination. The restriction map of these derivatives pGYOKI 15, 18 and 26 was also determined. In S. lividans all three determinants (Neo^r, $Thio^r$, Cm^r) were functionally expressed. Different deletion derivatives were isolated and characterized from the transformants.

STUDIES ON PLASMID DNA PATTERNS IN BACILLUS THURINGIENSIS STRAINS

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The main effective agent in commercial B. thuringiensis preparations used in many countries as biological insecticides is the so-called endotoxin. This protein has a high molecular weight and consists of only one polypeptide chain, i.e. it is coded by a single gene. If one attempts to improve the strains by genetic manipulation the knowledge of the location of this gene in the bacterial genome may be very useful. Recent results of several independent research groups suggested that the production of this protein may be connected with extrachromosomal DNA elements.

In our laboratory 12 B. thuringiensis strains - belonging to 8 different H-serotypes - were examined. Their plasmid pattern was investigated, then spo^-cry^+, spo^+cry^-, spo^-cry^- mutants and new derivatives cured of plasmid(s) were obtained from chosen strains. Changes in plasmid patterns caused by physical and chemical treatments (heat, NTG, ethydium-bromide, acridin-orange) were investigated. Comparing our results with others from the literature we concluded:

- most but not each of the strains contain extrachromosomal DNA element(s)
- the plasmid pattern of a strain does not relate to its H-serotype; it is characteristic only for the given isolate
- loss of plasmids or their total rearrangement may occur on physical or chemical effects or even spontaneously.

This great variability in the number and size of plasmids and published evidences on the presence of the toxin gene on the chromosome as well as on plasmid suggest a transposition-like phenomenon.

APPLICATION OF NOVEL GENETIC METHODS IN THE IMPROVEMENT OF ANTIBIOTIC PRODUCING MICROORGANISMS

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During the past 40 years the mutation programs have been proven to be successful in the improvement of antibiotic producing microorganisms resulting in 100-10,000-fold increase in productivity.

The discovery of genetic recombination in bacteria and fungi gave a good possibility to apply recombinants in the strain improvement (M. Jarai, Acta Microbiol. Acad. Sci. Hung. 8 73, 1961; M. Jarai, Hungarian Patent 151985, 1963; McDonald et al, J. Gen. Microbiol. 33 365, 1963). This method has not been applied generally because of the low recombination frequencies and the random combinations. The preparation of the recombinants by using bacterial and fungal protoplasts (Ferenczy et al, Nature 248 793, 1974; K. Fodor, L. Alfoldi, Proc. Natl. Acad. Sci. USA 73 2147, 1976), and the application of this method in Streptomyces (D.A. Hopwood et al, Nature 268 171, 1977) gave a new great chance to apply recombinants in the strain improvement.

In our experiments the recombinants of Micromonospora and Streptomyces strains have been produced by protoplast fusion. Some of these recombinants are both excellent starting strains in the improvement programs and overproducers. Mutation and selection steps are also applied continuously to improve the productivity of these recombinants.

The recombinant DNA techniques can also be applied in the improvement of antibiotic producing microorganisms as it will be shown by examples.

FUSION OF PENICILLIN-INDUCED ESCHERICHIA COLI PROTOPLASTS

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During the past few decades a substantial body of information on Escherichia coli accumulated and it enables us to use this microorganism as a model in the search for new methods. The exact genetic map of E.coli is known, so the genetic and biochemical interactions during and after the fusion process can be followed.

In removing the cell wall of the Gram(-) bacteria their double-layer membrane presented difficulties. Only one method produced some results: the inhibition of cell wall synthesis by penicillin. To reach a higher protoplast ratio I modified the methods known from the literature. By using adequate circumstances for intensive growth of the culture, aeration proved to be the most effective factor. It resulted more than 99 per cent protoplast yield.

In the system used for protoplast fusion PEG 6000 was applied; the yield of the fusion was investigated by direct selection. The most important result of the experiments is the production of recombinants; their frequency was correlated to the Taylor's genetic map of E.coli. There was only 10-15% deviation from the data of the genetic map, which could be attributed to the dispersion of the data of the experiments, but if this difference could be confirmed with other strains too, it might indicate a significant deviation of the results of the genetic mapping by fusion from the data obtained by earlier methods.

INVESTIGATION OF PROTOPLAST FORMATION AND REGENERATION OF MICROMONOSPORA PURPUREA

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Protoplast formation and regeneration have been achieved with wild type and mutant strains of M. purpurea. The optimal circumstances of the protoplast formation and regeneration of several M. purpurea strains have been studied.

The effects of different factors have been investigated: the conditions of growing, the concentration of lysozyme, the osmostabilizers, the combination of regeneration media. The conditions of protoplast formation of several strains have been established. The protoplast concentration of wild type and antibiotic resistant strains was higher than that of the auxotrophic mutants. The regeneration frequencies of several M. purpurea strains were found to be between 10-15%.

1/ D.A. Hopwood et al, Nature 268 171, (1977).
2/ R.H. Baltz, J. Gen. Microbiol. 107 93, (1978).
3/ Gy. Szvoboda et al, 5th I.Protoplast S., Szeged, 235, (1979).
4/ M. Frank et al, 15th FEBS, Brussels, 131, (1983).

PROTOPLAST FUSION IN MICROMONOSPORA SPECIES

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Protoplast fusion of filamentous bacteria has been carried out mainly with Streptomyces (D.A. Hopwood et al, Nature 268 171, 1977). Though Micromonospora species produce important antibiotics, very few genetic experiments have been done with them (Gy. Szvoboda et al, 5th I.Protoplast S., Szeged, 235, 1979; D.Y. Ryu et al, 4th I. Symp. GIM, Kyoto, 61, 1982). In our laboratory we have dealt with protoplast formation and fusion of Micromonospora species producing gentamicin, sisomicin and fortimicin (M. Jarai et al, 4th I. Symp. GIM, Kyoto, 108, 1982; M. Frank et al, 15th FEBS, Brussels, 131, 1983; M. Szigetvari-Gabanyi, M. Jarai, 6th I. Protoplast S., Basel, 361, 1983; M. Szigetvari-Gabanyi, M. Jarai, 9th Congr. Hung. Soc. Microb., 154, 1983; M. Jarai et al, 7th IBS, New Delhi, 102, 1984). Auxotrophic mutants and antibiotic resistant prototrophs have been applied to the preparation and fusion of protoplasts. The resistant prototrophs were found to be more useful for the strain improvement program than the auxotrophic parental strains. The recombination frequency with protoplast fusion was higher by two-three orders of magnitude than that with conventional crossing. The genetic analysis points out that the strains resulted by fusion can be true recombinants or parental segregants, the biparental feature is maintained in several generations. Since for the strain improvement only the stable recombinants are suitable, selective agents should be used for the isolation of recombinant strains after protoplast fusion.

THE ROLE AND IMPORTANCE OF CULTURE COLLECTIONS IN THE ERA OF BIOTECHNOLOGY

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The great developments and future prospects in the fields of genetic engineering and biotechnology have only strenghtened the fundamental role of culture collections in preserving, maintaining and supplying of microbial strains, plasmid, phages and cell lines for the purposes of research and application.

In fulfiling their basic tasks culture collections apply up-to-date methods of preservation and storage in order to guarantee the viability and unchanged genetic properties of strains. Beside these basic tasks culture collections should promote basic research (taxonomy, cytology, physiology) and training and education which will serve the further advancements in biotechnology.

Culture collections also have an indispensable function as depositaries for patented strains. According to the international rules being in force not only effective producing strains but also those having merit in the technique of genetic engineering can be patented and deposited.

The present state of culture collections in Hungary does not satisfy all requirements yet. Hence, within the frame of the national research program in biotechnology, an organization called National Collection of Agricultural an Industrial Microorganisms is being established that can further be developed for an international depositary for patented strains.

ASPECTS IN DEVELOPMENT OF MICROBIAL CULTURE COLLECTION

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A small culture collection was developed in the last ten years for education and research. The total number of maintained cultures is 549 including 115 bacteria, 332 fungi, 64 yeasts and 58 actinomycetes. As a subcollection nearly 100 thermophilic and thermotolerant fungi were isolated or collected by exchange.

Approx. 500 strains have been freeze-dried preparing 18 parallel ampules of each. Nearly 9000 ampules are stored in refrigerator at 5°C. For lyophilization an Edwards 30 PI/503 centrifugal freeze dryer (Edwards High Vacuum Ltd, England) was applied. The suspending medium contained (in 1:1 ratio) rabbit serum and pH 7 phosphate solution supplemented with inositol, ascorbic acid and cysteine · HCl. Good viability (survival) has been obtained, in per cent 96, 87, 91 and 88 for bacteria, fungi, yeasts and actinomycetes, respectively. We could not freeze dry some species of the following genera: Lipomyces and Sporobolomyces (yeasts); Alternaria, Ashbya, Colletotrichum, Eremothecium, Graphium, Helmithosporium, Sclerotium, Stemphylium and Verticillium (fungi). In general, many plant pathogenic fungi can not be lyophilized satisfactorily. The remaining strains are maintained on slant agars.

We are focusing on the isolation of further thermophilic microbes, which can be used in some biotechnological processes, like ensiling, composting or enzyme production.

A POSSIBLE MODEL OF THE CELL

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Improving the model of Monod, Perret and von Bertalanffy an environment-(substrate)-dependent model was constructed. Iconically it is a living system consisting of a membrane covered poikilochor living being and its relevant environment; with permeation, catabolic and anabolic processes in the former. The outside substrate (S_o) permeates and becomes inside substrate (S_i) and then its reaction sequence has two branches. It is irreversibly catabolized to inside product (P_i) and leaves the cell as outside product (P_o). Along the other branch S_i is reversibly anabolized to living material (L, incl. membrane), however, thermodinamically coupled to catabolism. Matematically: $S_i = k_1.L.S_o - /k_{-1}+k_2+k_4/.S_i+k_{-4}.L$; $P_i = k_2.S_i - k_3.P_i$; $L = k_4.S_i - k_{-4}.L$. Resolution I. (analytical) with constant S_o (continuous culture): $S_i = \Sigma \alpha_i.e^{\lambda i.t}$; $P_i = \Sigma \beta_i.e^{\lambda i.t}$; $L = \Sigma \gamma_i.e^{\lambda i.t}$. Resolution II. (simulation) with decreasing S_o (batch culture): Values of S_o, S_i, P_i and L change with time according to the curves of growing microbes (e.g. E.coli). Moreover the system responds to sudden changes of the environment, viz. increase or decrease of substrate concentration, after a lag period and in a logical sequence of changes of the above parameters, exactly like E.coli. Besides, the lag phase at the beginning of culturing is, at least partly, of the same origin.

1/ C.J. Perret, J. Gen. Microbiol. 22 58, (1960)
2/ L. von Bertalanffy, Science 111 23, (1950)
3/ J. Monod, Ann. Rev. Microbiol. 3 371, (1949)

THE EFFECT OF CO_2 ON CELL PROLIFERATION

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Observations related to the regulation of the cell division cycle can be explained by supposing an intracellular oscillation process. Lately we have developed a mathematical model leading to a limit-cycle type oscillation based on CO_2 production by the Krebs-cycle and CO_2 fixation in anaplerotic reactions.

In order to provide experimental support we have studied the incorporation of ^{14}C labeled HCO_3^- during the cell cycle of heterotrophic microorganisms (C. tropicalis, E.coli). Microbes synchronised by nutrient starvation were cultivated in an aerated, pH controlled 100 cm^3 fermentor in the presence of $H^{14}CO_3^-$ and ^3H-thymidine. The following parameters were measured:

1/ ^3H-thymidine incorporation by liquid scintillation (Rack Beta, LKB - Wallac).

2/ Cell density by electronic particle counter (Laborscale Medicor).

3/ Incorporation of ^{14}C into cells by liquid scintillation (Rack Beta, LKB - Wallac).

4/ $^{14}CO_2$ activity in the exit gas by an anthracene filled detector (Tri-Carb Scintillation Spectrometer, Packard).

5/ Dissolved O_2 concentration in the medium by an O_2 electrode (Radelkis).

6/ O_2 and CO_2 concentration in the exit gas (Blood Gas Analyser, Radelkis).

From the measured data the specific activity of $^{14}CO_2$ in the cell can be deduced and the real rate of CO_2 fixation can be calculated after correcting the ^{14}C incorporation data by the specific activity of $^{14}CO_2$.

ANAEROB PHYSIOLOGY

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We should accept the definition of on anaerob as "an organism incapable of growing in air, receiving its essential oxygen from a source other than the atmosphere" (Cowan, 1968). Using enzymological criteria, anaerobes are organisms that do not posses either cytochrome oxidase or oxigenases and as a consequence of these, they generate energy and synthesize their substances without recourse to molecular oxygen. Though anaerobic bacteria can use a variety of growth substrates, their distribution in nature is determined by the availability of those organic and inorganic electron donors and acceptors that they utilize for energy generation. One-carbon compounds at all oxidation levels between methane and carbon dioxide occur throughout nature and they are also evolved as endproducts of many anaerobic fermentations. Organic nutrient-rich anaerobic locations are very rich in fermentative species. The purpose of all these fermentations is the concurrent net synthesis of ATP which is accomplished by substrate level phosphorylation reactions. During the last years evidences have been accumulated that many anaerobic bacteria can synthesize ATP via electron transport phosphorylation previously thought to occur only in chemotrophic aerobes. In view of many new developments it seems appropriate to bring up to date what is known about metabolism and energy transformation of this group of organisms.

EFFECT OF NYSTATIN ON THE IONMOVEMENTS OF CANDIDA ALBICANS

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Minimal growth inhibitory concentration (MIC = 6 $\mu g.ml^{-1}$) of Nystatin (Nys) and the membrane desintegrator cetyl pyridinium bromide (CPB = = 16 $mg.ml^{-1}$) inhibit the $^{45}Ca^{2+}$ (10^{-3}, 10^{-4} M) influx by 86% and 98.5%, resp. The spontaneous Ca^{2+}-efflux (63% in 50 min) of preloaded cells was not influenced by Nys, while CPB caused 96% leakage in 50 min. Resting cells did not take up, within 50 min, considerable amounts of 0.13 M arg, lys and his, while 0.05 M leu and 0.3 M gly penetrated easily. MIC Nys did not influence leu influx in 50 min, but 100 $\mu g.ml^{-1}$ after 10 min not only inhibited the further uptake, but decreased the previous control level (30th min: 66%, 50th min: 35% of control). MIC and 100 $\mu g.ml^{-1}$ Nys inhibited gly influx in 50 min only by 33% and 67%, resp., while CPB inhibited it completely. Simultaneous addition of 10^{-3} M Ca^{2+} and gly did not influence the uptake of the latter, however, it prevented the inhibitory effect of MIC Nys on the gly uptake.

According to the above data and our previous results (I. Berek, E.K. Novak, Abstr. Intern. Symp. Wirkungsmech. Fungiziden, Antibiot. Cytostat. (eds. H. Lyr, W. Rawald) Akad. Verlag, Berlin, p.173-181, 1970; I. Vincze, J. Zala, E.K. Novak, Proc. 21st Hung. Annu. Meet. Biochem. Veszprem, p.219-220, 1981), not only the discriminating ability of the plasmamembrane, but also some uptake processes persisted after a treatment with the biologically relevant (MIC) concentration of Nys. Thus the antifungal effect of Nys cannot be explained simply by its membrane-pore inducing ability.

PHAGES OF BACILLUS LICHENIFORMIS AND BACITRACIN PRODUCTION

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The purpose was to improve the Bacillus licheniformis strain used for bacitracin production. The industrial strain was shown to be lysogenic. From the induced lysate three phages were isolated and characterized by electron microscopy and infection specificity. The phages were found temperate and heteroimmune as shown with the prepared, partially cured lysogenes and confirmed with selective hosts.

Bacitracin production is independent from lysogeny as demonstrated by cured strains and reconstructed lysogenes, but spontaneous induction of phages followed by lysis of the bacteria interferes indirectly with the fermentation.

Virulent phages of B. licheniformis were also isolated and characterized. The infection by these phages prevents antibiotic production through the lysis of the cells.

For industrial purposes the construction of less inducible and virulent phage resistant strain was attempted. Partially cured lysogenes were reinfected with mutant phages to introduce erroneous temperate regulation and exclusion of virulent infection. Virulent phage resistant, bacterium surface mutants were also isolated.

To increase antibiotic production of the strains, random mutation and selection, antimetabolite action and differential heat sensitivity of cell or phage growth and bacitracin synthesis was applied.

MICROBIAL PRODUCTION OF LYSINE

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Some essential amino acids (lysine, tryptophan, methionine) are less frequent in plant proteins than in animal proteins, therefore their addition has a particular importance in animal nutrition. Every million tons corn seeds require about 1-3000 tons supplement of lysine. The import of lysine in Hungary was 2450 tons in 1983. The bulk of lysine throughout the world is produced by microbial processes. Like in any other industrial process, economic aspects are of primary importance in choosing the raw material for a fermentation process. The principal aim of investigations has been to determine an optimum and cheap medium for high lysine production from several raw materials, using some special auxotrophic and regulatory mutants. In the industrial production of lysine molasses, starch hydrolysate (hydrol) and corn hydrolysates can be used as carbon sources. The highest amount of lysine (100 g/l) was achieved in a medium containing hydrol. A solid feed-concentrate of lysine was made by evaporation and spray drying of the broth.

UTILIZATION OF FURFURAL-PROCESS-WASTE BY MICROBIOLOGICAL AND ENZYMATIC METHODS

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Over the past few years extensive research has been directed at the utilization of renewable resources and wastes for energy supply, as well as for the production of chemicals, food and feed products. In spite of all efforts, however, no satisfactory solution has been found so far.

The aim of our work was the utilization of the furfural-process--waste, first as the carbon source of cellulase fermentation, second as the substrate of the enzymatic hydrolysis.

The enzyme fermentation was carried out in a 10 l stirred, aerated fermentor at 30°C, the maximum concentration of the furfural-process-waste was 40 g/l, the reached enzymatic activity in 75 hours was 2,4 IU/ml FPA (Mandels, 1976). We used the culture filtrate directly in the hydrolysis of the furfural-process-waste at 50°C, pH 4.8.

At 13% substrate concentration, with an enzyme/substrate ratio of 13.3 IU/g, 73% conversion (calculated for total carbohydrate) was obtained in 25 hours.

The broth obtained by enzymatic hydrolysis, containing 30-40 mg/ml reducing sugar, was utilized by different fungi and yeasts. In the case of Agaricus campestris and Trametes versicola, the production was performed directly, without the removal of the solid, undecomposed residue, and in the case of yeast the supernatant was used only. In the latter case the sugar utilization of Candida guilliermondii and Candida tropicalis was 92-94%, the amount of yeast gained from 1 g sugar was 0.5 g, and the raw protein content of the yeasts amounted to 35-40%.

M. Mandels et al, Biotechnol. Bioeng. Symp. No.6. 21, (1976)

EXPERIMENTS TO IMPROVE PECTIN LYASE PRODUCTION OF ASPERGILLUS FOETIDUS

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The aim of this work was to increase the pectin lyase (PL) production of Asp. foetidus at the expense of its pectin esterase synthesis by varying the composition of the nutrient medium, with special respect to the ratio of carbon and nitrogen sources.

Experiments have been carried out in 10 liter fermentors at 28°C. Agitation speed: 460 rpm, aeration rate of 1.5 $1.1^{-1}.min^{-1}$. As carbon and nitrogen sources dried sugar beet slices and $(NH_4)_2SO_4$ were used, resp.

In agreement with the results of our previous kinetic analysis (N.X. Thien, K. Zetelaki-Horvath, 1984) demonstrating that the pectin lyase synthesis of Asp. niger was growth associated, a tight correlation was found betwwen the mycelium and pectin lyase production of Asp. foetidus cultures, too.

Pectin lyase synthesis of Asp. foetidus was influenced by the ratio of carbon and nitrogen sources of the medium, and it was increased significantly when the ratio of carbon and nitrogen sources was 5.7.

The highest activity was attained in the 140 h culture (3.48 μmol. $.min^{-1}.cm^{-3}$, which is the mean value of 9 fermentations of 3 consecutive runs, and among the results, activity over 5 was found), but it did not differ significantly from that obtained in the 120 h culture (3.3 μmol. $.min^{-1}.cm^{-3}$). Next to the high PL activity, the use of this medium resulted in a very low pectin esterase activity (0.20 mval.$min^{-1}.cm^{-3}$) too.

Nguyen Xuan Thien, K. Zetelaki-Horvath, Acta Alimentaria, In Press, (1984)

EFFECT OF A NEW, SYNTHETIC, CYTOKININ-LIKE REGULATOR ON THE TISSUE CULTURE AND ALKALOID PRODUCTION OF DATURA INNOXIA

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A new, synthetic, cytokinin-like regulator: N,-n-isopropyl--benzimidazolium-chloride(Sz-28) was developed by the Institute of Isotopes and North-Hungarian Chemical Works, which has a remarkable stimulating effect on the protein synthesis, photosynthetic $^{14}CO_2$ fixation, chlorophyll-preservation in senescing leaf tissue. The regulator-induced stimulating effect on protein synthesis parallels the retardation of decrease in chlorophyll level.

The effects of Sz-28 regulator have been studied both on the biomass and alkaloid production of the callus culture induced from Datura innoxia Mill. roots. Adding Sz-28 regulator (instead of the usual component, kinetin) the increase (in dry weight) was 30% and 60% for the biomass and alkaloid production, respectively.

When one of the biosynthetic alkaloid precursors, phenylalanine was added with Sz-28 to the culture medium the effectivity of the new regulator on the tropane alkaloid synthesis was doubled. The results were proved by isotopic estimations, too. Incorporation of radioactivity into the quaternary alkaloid fraction was significant (65% increase over the control material).

1/ N. Hiraoka, Studies on alkaloid production in Datura tissue cultures. Dissertation, Kyoto (1976).

2/ B.I. Pozsar, T. Szarvas, M. Maroti, Bot. Kozlem. 67 313-314 (1980).

3/ E. Szoke, N.N. Dung, G. Verzar-Petri, A. Potoczki, Acta Bot. Acad. Sci. Hung. 29 403-410 (1982).

COMPARATIVE STUDIES ON SOLUBLE AND IMMOBILIZED RABBIT MUSCLE PYRUVATE KINASE

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Pyruvate kinase (EC 2.7.14) was isolated from rabbit muscle. For the preparation of immobilized enzyme a polyanionic type support Akrilex C was used. The activation of the gel was carried out by water soluble carbodiimide according to B. Szajani (1980), and 180-200 U/g_{solid} bound enzyme activity was achieved.

Comparing the pH optimum of soluble and immobilized enzyme a shift was observed in alkaline direction. The apparent temperature optimum of enzyme activity was found at about 35°C for the soluble pyruvate kinase and that was for bound one between 34 and 42°C. The apparent K_M value for phosphoenolpyruvate increased to 0.8 mM, and the K_M value for ADP was found to be 0.42 mM, i.e. less than that of the soluble enzyme (0.52 mM).

The heat stability of bound and soluble enzyme was studied in triethanolamine/HCl buffer at pH 7.5 between 45 and 65°C. The bound enzyme seemed to be more stabile against heat inactivation. The pH stability was measured at pH 7.8 and 9. There was no significant difference in pH stability between the soluble and immobilized enzyme. The stability was highest at pH 7, where after 4 days about 60% activity remained. The stability against urea was determined in the presence of 0.1-3.0 M urea at pH 7.5. At low concentration of urea the immobilized enzyme was found to be more stable, however, after 10 min incubation in the presence of 3 M urea only 10% of the activity was measured for both enzymes.

B. Szajani, Acta Biochim. et Biophys. Acad. Sci. Hung. 15 295, (1980)

PURIFICATION AND PROPERTIES OF A CYCLODEXTRINASE FROM BACILLUS MACERANS MNG00154

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A novel enzyme has been isolated from filtrates of 5 days old submerged cultures of Bacillus macerans MNG00154 strain. The enzyme has been purified by affinity chromatography on alpha cyclodextrin-Sepharose followed by DEAE-cellulose and Sephadex G-75 gel filtration chromatographies and chromatofocusing.

The enzyme is able to hydrolyze cyclodextrins to maltose without cyclizing them into another cyclodextrin homologues, thus it can easily be distinguished from the well-known cyclodextrin glycosyltransferase (E.C.2.4.1.19) which is also produced by this strain.

The purity of the enzyme has been tested by SDS-PAGE and also by agarose slab IEF. By both methods a molecular weight of 68,000 and an isoelectric point of 4.65 have been estimated. The optimum pH of its action on alpha cyclodextrin is 6.2, the same as the value observed for potato amylopectin.

The calculated K_m and V_{max} values are the greatest with alpha and beta cyclodextrin substrates and they are decreasing in the order maltotetraose - maltotriose - DP16 short chain amylose - potato amylopectin - rabbit liver glycogen.

HYDROLYSIS EXPERIMENTS WITH THE COMPONENTS OF A CELLULOLYTIC ENZYME PREPARATION

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The components of a crude enzyme preparation from the strain Gliocladium were separated by preparative isoelectric focusing and analysed for enzyme activity.

The investigations confirm the results obtained by other authors (L.G. Fagerstam, L.G. Petterson, FEBS Lett. 98 363-367, 1979) namely that the enzymes in the complex can be divided into three groups: endoglucanases, exoglucanases or cellobiohydrolases, and cellobiases or -glucosidases. The components were isolated and characterized with regard to their activity towards the different substrates. The molecular masses were in the range 12,000-65,000, and the isoelectric points were between 3 and 9.

The enzyme preparation was tested in numerous hydrolysis experiments with Avicel as a substrate, at optimal pH and temperature conditions, pH 5.0 and 50°C, respectively. The enzyme alone, even at high concentrations, can hydrolyse 10% of the cellulose only, but if cellobiase is added, approximatively 25% hydrolysis can be obtained. This indicates that cellobiose is an inhibitory product, and that its effect can be decreased by the addition of cellobiase.

EFFECT OF ENZYMATIC PROCESSING ON THE PECTIN CONTENT OF FRUIT AND VEGETABLE NECTARS

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Up to date and economic production of fruit and vegetable juices can be carried out with the use of special enzymes. Endo-polygalacturonase (endo-PG) is of great importance in the fruit and vegetable processing. It decomposes the protopectin containing middle lamella of plant tissues, resulting in cell suspensions consisting of single cells, cell agglomerates and soluble pectins.

Pectin is a valuable substance from the point of view of health and nutrition physiology (K. Zetelaki-Horvath et al, Elelmezesi Ipar 37 412, 1983). It was determined (by the carbasol method, Pressey et al, J. Food Sci. 36 1070, 1971), in the juices before and after enzyme treatment in pilot and industrial scale.

Various fruits and vegetables were treated by Phylendonase (endo-PG, Phylaxia, Budapest, activity: SPA_{75}^{Na-p}: 300 $l.h^{-1}.cm^{-3}$). Treatment of various fruits and the squash was carried out by 0.5 and 5 $cm^3.l^{-1}$ endo-PG at 40 and 45°C for 20 and 40 min.

After enzyme treatment the pectin content of apricot, peach, plum, quince apple, sour cherry and squash juices increased by 4.5, 1.1, 1.2, 2.0, 1.0 and 1.5 $mg.cm^{-3}$, respectively.

Results of pilot and industrial scale processing showed that the traditional heat treatment decreased the pectin content of the juices considerably (by 30 per cent). As pectin has many beneficial effects in the human organism, the use of flash pasteurization can be suggested for the preservation of pectin, vitamins and colour as well.

ENZYMES IN SOLUBILIZING AND PRESERVING COLOURS OF RED BEET

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Fibrous fruit and vegetable juices are of great importance from the point of view of nutrition physiology, therefore our aim was to produce vegetable cocktails with low energy content. As basic component red beet (having high protein and mineral content, as well as a pleasant colour) was chosen. Among vegetables red beet is the richest source of Mg. Its consumption is supposed to result in higher resistance of the human organism against different illnesses.

Before cocktail production 3 red beet varieties (Detroit, Bordo OMFI and Biborhenger) were treated with endo-polygalacturonase and pectin lyase (K. Zetelaki-Horvath et al, Hungarian Patent 176 825, 1981; K. Zetelaki--Horvath, Acta Alimentaria 12 11, 1983). Betacianin and vulgaxantin content of the obtained juices was tested (T. Nilsson, Swedish J. Agric. Res. 3 187, 1973).

Best colour (betacianin 360 and vulgaxantin 140 $mg.l^{-1}$) was attained in the juice of Detroit (endo-PG: 0.75 $cm^3.100\ g^{-1}$ red beet). To obtain a cocktail of low energy content, red beet juice was mixed with juices of other enzyme-treated vegetables, like red paprika (high vitamin C and beta-carotene content) and Jerusalem artichoke as a natural sweetener (containing inulin and fructose).

Colour stability of these cocktails was tested over a 6-month storage period, in natural state, with or without an anti-oxidant (glucose oxidase).

The use of glucose oxidase (20 $mg.100\ cm^{-3}$) in the red beet cocktails resulted in a 30 per cent higher colour stability compared with that of the controls.

SEMI-INDUSTRIAL APPLICATION OF ULTRAFILTRATION IN ENZYME-PROCESSING

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It is well known that ultrafiltration equipments can be used in biotechnology in a number of fields, e.g. elimination of inhibitory products from the culture medium, cell recycling in different fermentation technologies, purification of dissolved end-products. The advantages of ultrafiltration: it can be carried out under sterile circumstances, it can be applied to heat-sensitive materials, besides its concentrating effect purification effect is also significant, finally it doesn't need much energy. The possibility of concentrating some enzymes produced by PHYLAXIA has been investigated using an equipment built for laboratory work. It was equipped with different membranes made by ABCOR. Three membrane-types were tested (HFM-180; HFM-100; HFK-131) with different enzymes (cellulase, pectinase, amilo-glycosidase, rennin, pectine-lyase, phylendonase). Based on these tests the membrane type HFM-100 has been chosen for the semi-industrial equipment. The construction has been carried out by GEPSZEV. The total filtration surface of two mounted spirals is 10 m^2. During the ultrafiltration tests with the above mentioned enzymes, the decrease of filtration speed with time, decrease of enzyme activity and the concentrating effect were tested. Max. decrease of activity was 20% at 10-15-fold concentration. The speed of concentration was between 0.15-0.25 m^3/h. Energy-demand of the equipment was 5.5 kW/h.

COMPARATIVE STUDY OF PROCESSES FOR RECOVERY OF CEPHALOSPORIN C BASED ON ADSORPTION AND ION-EXCHANGE

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Processes applying adsorptive and ion-exchanger resins for the recovery of cephalosporin C are quite commonly used. In the lecture the results of a comparative study of these resins (produced and marketed by different companies) are shown. The resins were compared from the points of view of loading capacity, separation of cephalosporin C and desacetylcephalosporin C, width and substance concentration of the elution peak and possibilities for regeneration. The possibility of obtaining cephalosporin C salts without the application of the ion-exchanger was also examined; the properties and behaviour of this product in the course of cleavage were studied.

SEPARATION OF GENTAMICIN C COMPLEX COMPONENTS

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Separation of gentamicin C complex components is quite important both for analitical and preparative purpose. The lecture gives an overview of methods and processes applied for these purposes, including the Craig extraction process, column-chromatographic methods using different types of stationary phase (e.g. cellulose, cellulose phosphate, silicagel, cation-exchanger resin etc.), HPLC methods. The new HPLC method suitable for the separation, detection and quantitative determination of all the five components is communicated, too.

APPLICATION OF A MICROPROCESSOR CONTROL SYSTEM IN FERMENTATION

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The use of computers in fermentation is a widely discussed topic. The task of such a system is to optimize the parameters in the course of the development of a fermentation process.

Once a technology is established, the operation of the fermentation is usually performed by conventional analogue instrumentation. In case of a computer connection the system allows the collection, handling and logging of data as well as supervisory control.

Some advanced tasks, i.e. the control of a certain fermentation variable or an operation, finally the control of the whole fermentation process can be performed automatically by using a microprocessor-based controlling system.

The lecture describes such a microprocessor controlled design (based on Hung. Patent - Zentay et al, prior. 788, 1981). It is permitting safe fermentation runs, intelligent dosing possibilities besides the improved measurement, control and registration of the usual fermentation parameters. Characteristic features of the construction include the use of direct digital control (by which the need for separate analogue instruments is eliminated) and free memory capacity by which, among others, time-based control of many steps and complex dosing programs can be applied.

Preliminary experiments of the application of the microprocessor system will be presented.

SCALING UP OF ANTIBIOTIC FERMENTATIONS

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Biotechnologists agree that in the scaling up of antibiotic fermentations one of the most important measures is the maintainance of the volumetric oxygen transfer coefficient (K_La) at the same level in the different size fermentors (M. Jarai, Proc. IV. IFS: Ferment. Technol. Today, Kyoto, 97, 1972; M. Jarai, Magyar Kemikusok L. 32 129, 1978).

In our experiments on the scale up of the fermentations with filamentous microorganisms we have paid great attention to the K_La decreasing effect of the highly viscous, non-Newtonian fermentation broth. Therefore during the fermentations we have decreased the viscosity of the broth increasing by this way the K_La.

It will be shown that the maintainance of the tip speed of the agitator at the same level in the different size (300 l, 150,000 l) fermentors is a very useful scale up parameter. Dissolved oxygen can be kept at a suitable concentration by changing the air flow rate, or by the dilution of the broth. By avoiding the increase of the tip speed the shear stress does not surpass the critical level.

MULTICHANNEL ANALYSER SYSTEM WITH QUADRUPOLE MASS SPECTROMETER

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The mass spectrometer of the system works in the range of 1-300 atomic mass unit; multicomponent analysis of the samples can be performed. Conventional detectors are specific, while mass spectrometers are able to detect many components.

It is the sampling system which makes possible to analyse many samples with the same MS. Probes with semipermeable membranes are immersed into the liquids of fermentors and into the flow of exhaust gases and laboratory samples as well. Gases dissolved physically and free gases are measured on-line, and fixed components can be measured off-line after chemical or biological treatment. Response time of the membrane probe is about 20s, and the time-lag between two successive probes is 1-5 min, depending on the dimensions of the tubing and the type of gas to be measured. Free gases can be measured with max. 0.1s response time with a special two-step sampling system.

The whole system is controlled by a microcomputer unit. The important parameters (mass numbers, sensitivity, number and sequence of probes etc.) can be programmed on its keyboard before and during the measurement. The actual values are displayed on the monitor in analog, linear bargraph, logarithmic bargraph or table mode. 8 analog outputs are available for 8 programmable components and digital data transfer to the host computer is available for further data acquisition.

DETERMINATION OF NON-VOLATILE COMPONENTS IN FERMENTATION BROTH BY MASS SPECTROMETER

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The Quadrupole Mass Spectrometer is a reliable device for measuring gases and volatile compounds that can permeate through a thin membrane, whereas non-volatile compounds, i.e. glucose, saccharose, amino and keto acids, and carbonates cannot be measured directly. In the case of some keto acids the esterification seems to be a proper method for increasing the volatility of the molecules.

A method was developed for measuring glucose, saccharose, aminonitrogen and carbonates contained in fermentation broth following gas producing chemical reactions.

Earlier the gasometric methods were widely used in quantitative analyses. These methods were originally rather complicated, therefore they have been replaced by other ones. The QMS enables the application of the conventional methods in a new form which is much simpler and quicker than the old ones.

This new method measures the generated gas in a continuous liquid stream and can be applied for serial analyses.

QUADRUPOLE MASS SPECTROMETRIC ANALYSER AND CONTROL SYSTEM FOR MONITORING INDUSTRIAL FERMENTATION PROCESSES

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A microprocessor controlled quadrupole mass spectrometer system equipped with a sampling unit has been developed for the measurement of gases and volatile components involved in the fermentation processes.

The exhaust gases of the fermentors and some components dissolved in the fermentation broth (e.g. O_2, CO_2, N_2, propanol) were analysed on-line and filtered samples were taken for off-line determination of NH_3, pyruvate, 2-keto-glutarate, phenylacetic acid, HCO_3^-, and glucose contents. Samples from different probes were analysed quasi-simultaneously.

The concentrations measured were in close correlation with the physical parameters of the fermentations (agitation, temperature, aeration) and the biological state of the microorganisms. The results were compared with conventional analytical methods, too.

The result of this research and development program is a complex analyser-control system which can be used for laboratory research purposes and for the control of industrial fermentation.

FINAL PURIFICATION OF BIOLOGICAL PRODUCTS

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The final purification of solutions produced by bioreactors is necessary to obtain useable products e.g. pharmaceuticals.

Earlier methods included various precipitation processes. These, however, besides the considerable amount of manual work could also entail low yield, denaturation etc. They are now being gradually superseded by chromatographic methods. Low pressure liquid chromatography is the one most frequently used, though industrial high pressure liquid chromatographic methods are also introduced (Waters).

The main liquid chromatographic methods are molecular sieving or gel filtration, ion exchange and affinity chromatography.

Some examples of their use in Hungary are the purification of Gordox with Sephadex G-50 gel, the purification of interferon with CPG (controlled pore glass), the obtaining of plasma proteins with ion exchangers (DEAE Sepharose, CM-Sepharose) and that of antithrombin III and fibronectin with affinity chromatography.

One of the advantages of these methods is their easy automation. With the help of relatively simple pumps, magnetic valves and detectors these electronically controlled equipments automatically regenerate the columns, apply and elute samples. An important point is the quality of the columns, which must be tested regularly.

For the time being, ultrafiltration is most suitable for the separation of macro and micromolecular solutes (desalting), as well as the concentration of macromolecular solutions. On the other hand, the industrial application of ultrafiltration for the separation of macromolecules is still far from perfect.

Special attention must be paid to keep these products pyrogen-free (pharmaceuticals). This can be assured only with aseptic conditions and continuous microbiological control.

ANTIBIOTIC PRODUCTION OF PROTOPLASTS

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Earlier experimental facts indicate that in the Streptomycetes strains there is some connection between the biosynthesis of the cell wall and that of the aminoglycoside antibiotics. We wanted to study this problem further, therefore the antibiotic production of the protoplasts and that of the mycelium were compared. These experiments were carried out with a streptomycin producer Streptomyces griseus strain.

For measuring the antibiotic production of the protoplasts and the control mycelium, we used ^{14}C labeled myo-inositol, which is a good precursor molecule of the streptomycin. In the course of our experiments we compared the streptomycin production of the protoplast, that of the mycelium, the immobilized mycelium and the immobilized protoplasts. The immobilization was carried out using Ca-alginate gel.

Our results suggest that the protoplasts are able to produce streptomycin, and the incorporation of ^{14}C-myo-inositol into streptomycin is higher in the protoplasts than in the mycelium. The protoplast culture, having the same protein content as the mycelium, incorporated 19% whereas the mycelium incorporated only 5% of the radioactive precursor. The immobilized protoplasts also produced more ^{14}C labeled streptomycin than the immobilized mycelium.

SYNTHESIS AND BIOLOGICAL ACTIVITY OF SOME NEW N-PHOSPHONO-ACETYL-DIPEPTIDES

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The enkephalinase (Ease) and the angiotensin-converting enzyme (ACE) cleave the Gly^3-Phe^4 bond of enkephalins. For inhibition of Ease and ACE a lot of derivatives of Ala-Pro (E.D. Thorsett, Proc. Natl. Acad. Sci. 79 2176, 1982) and Phe-X (M.C. Fournie-Zaluski., J. Med. Chem. 26 60, 1983) have been synthesized. Optically active N-phosphono-acetyl (PA, i.e. /$HO/_2P/O/CH_2CO$-) dipeptides in L-configurations were prepared in our laboratory: PA-Ala-Pro-OH/1/, PA-Ala-Hyp-OH/2/, PA-Ala-p-Glu-OH/3/, PA-Phe-Gly-OH/4/, PA-Phe-β-Ala-OH/5/, PA-Phe-Gaba-OH/6/, PA-Phe-Ala-OH/7/, PA-Phe-Leu-OH/8/.

The compounds were characterized by paper-electrophoresis, TLC as well as IR, NMR spectra. The biological activity of the compounds was investigated in vivo. Antihypertensive effects were measured in conscious, spontaneously hypertensive rats. Analgesic effects were studied in hot plate tests.

Compounds (1-3) were administrated p.o. in 50 mg/kg doses to rats. Hydralazin and captopril were used as reference compounds. (1-3) had a mild inhibitory effect on ACE. The blood-pressure of rats was decreased by 20%. The activities of these compounds reached a maximum 15-30 min after their administration and lasted for about one hr. The heart rate was unaltered. (1-3) showed no inhibitory effect against Ease in the enzyme assay. (4-8) given p.o., led to dose related analgesia in rats. This effects was blocked by naloxone. There was no antihypertensive effect. Compounds (1-3) and (4-8) showed different interactions with Ease and ACE.

DISSOCIATION OF THE ANTIAGGREGATORY EFFECT FROM THE ADENYLATE CYCLASE STIMULATING POTENCY OF THE SELECTIVE VASODILATOR PROSTACYCLIN ANALOGUE: 5-NITRO-PGI_1

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The purpose of the prostacyclin analogue research is to reach higher antiaggregatory and less hypotensive activity. 5-nitro-PGI_1 is a derivative of reverse selectivity (P.Sz. Kormoczy et al, Mol. Cell. Cardiol. 15 (Suppl.1) p.96, 1983). A causal relationship has been reported between the antiaggregatory and platelet adenyl cyclase (AC) activating potency of prostacyclin (Platelets in Biology and Pathology 2 217-220, Elseviers 1981, ed. J.I. Gordon).

5-nitro-PGI_1 has negligible antiaggregatory activity. Its IC_{50} on human PRP (2 μM ADP) is above 100 μg/ml (prostacyclin: 2 ng/ml). However, the human platelet AC activating potency of 5×10^{-7} M PGI_2 and 5-nitro--PGI_1 is 442 ± 66% and 312 ± 59% respectively. Thus 5-nitro-PGI_1 is more than half as potent as PGI_2. Our data do not suggest a simple relationship between the two parameters.

INHIBITORY EFFECT OF SYNTHETIC PROSTACYCLINE DERIVATIVES ON PLATELET FUNCTION IN "EX VIVO" EXPERIMENTS

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Prostacycline - the most powerful inhibitor of platelet aggregation - can not be used widely since its biological half-life is very short, and it is liable to chemical hydrolysis. The purpose of the research in this field is to produce chemically and biologically stable derivatives of prostacycline. 7-oxo-PGI_2 (Kovacs G. et al, J. Med. Chem. 25 105, 1982) and 13,14-didehydro-20-methyl-meta-interphenylene-PGI_2 (BS-606) (Szekely I. et al, 2nd Int. Conf. on Chem. Biotechn. of Biol. Act. Nat. Prod. 15-19, 08.1983. Budapest, A-83) are hydrolitically and biologically stable analogues. They showed considerable antiaggregatory activity at "in vitro" experiments according Born (Born, G.W.R., Nature 194 937, 1982). IC_{50} values were: 3.6 mg/ml for PGI_2; 224 ng/ml for 7-oxo-PGI_2; 104 mg/ml for BS-606 on rabbit platelet rich plasma induced by ADP. Compounds were tested by the modified "whole blood" method (Wu et al, Lancet 294 1974). IC_{50} values were in this case: 6.1 ng/ml for PGI_2; 224 ng/ml for 7-oxo--PGI_2; 86.3 ng/ml for BS-606. The question investigated in the "ex vivo" experiments was whether the stabilized prostacycline derivatives have antiaggregatory activity after oral administration. The orally administered 7-oxo-PGI_2 was found to have antiaggregatory effect by the "whole blood" method, while PGI_2 and BS-606 had no activity in this experiments. After intravenous administration each compound showed considerable activity (0.67 ng/kg/min, 41.6 µg/kg/min and 14.6 µg/kg/min were the IC_{50} values for PGI_2, 7-oxo-PGI_2 and BS-606, respectively). These compounds had an influence on platelet 3-factor availability. An increased cAMP level could be observed in the blood after administration of the compounds. These results suggest that 7-oxo-PGI_2 is an orally active antiaggregatory prostacycline derivative; it was stabilized against chemical hydrolysis; the duration of its activity was a bit longer than that of prostacycline since its metabolism was not so fast.

INHIBITION OF THE FORMATION OF HYDROXYARACHIDONIC ACID DERIVATIVES PRODUCED BY LIPOXYGENASE

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It is a widely accepted fact that the SRSA - a mixture of leukotrienes (LTC_4 and LTD_4) - is an important mediator of asthma. Leukotrienes are the products of specific lipoxygenase enzymes which belong to the family of arachidonic acid oxygenase enzymes.

The purpose of the current research on antiasthmatics is to find compounds which antagonize the effect of leukotrienes on receptor level, or inhibit their biosynthesis. Smith has reported that 6,9-diepoxy-6,9--(phenyl amino)-6,8 prostaglandin I_1 (marked U 60257) has an inhibitory effect on leukotrien activity and biosynthesis (Smith et al, Prostaglandins 24 (4) 43, 1982). One of the Chinoin's research programs is closely connected with the above mentioned field. Therefore, we prepared the U-60257 as a standard. This compound was investigated in different biochemical and pharmacological tests: inhibition of different lipoxygenases and glutathion transferase enzymes; antagonism with SRSA on guinea pig ileum. Our results were as follows. U-60257 inhibits the lipoxygenase enzyme, IC_{50} = 3.0 μM for the human leukocyte lipoxygenase, IC_{50} = 10 μM for potato tuber and 200 μM for soybean lipoxygenase. An inhibitory effect can be observed on glutathion transferase, IC_{50} = 67 μM. U-60257 antagonized the activity of LTD_4 on guinea pig ileum, IC_{50} = = 2.1 μM. It seems that U-60257 has a considerably specific inhibitory effect on lipoxygenase. Moreover, the results indicated that the compound acts in two ways: it inhibits the SRSA producing enzymes and it antagonizes the effect of SRSA on receptor level.

ANTICOAGULANT ACTION OF A SYNTHETIC COPOLYMER WITH SULPHATE AND CARBOXYL GROUPS

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The effects of the 3800 mol.wt. copolymer containing 24 mol% sulphate and 8 mol% carboxyl groups were studied on haemostasis. In vitro experiments indicated that this copolymer (CP) inhibited blood coagulation by influencing the kinetics of thrombin inactivation by antithrombin. The in vivo anticoagulant activity of the CP in rats was monitored using whole blood clotting time (WBCT). The prolongation of WBCT after i.v. administration of 5, 10, 20 and 40 mg/kg doses of CP was dose dependent and was seen over periods of 60 to 180 minutes. According with their different in vitro effects, CP and heparin (HEP) possessed different activities also in vivo. A comparable prolongation of WBCT was observed at 90 and 180 minutes after the application of 20 mg/kg CP or 150 IU/kg HEP. However, the magnitude of changes produced by CP at 30 and 60 min was significantly less than that induced by HEP. In dogs during a 180 min period the max. inhibitory activity of 50 IU/kg HEP was superior to that of 10 mg/kg CP, although the CP showed more prolonged effect. The relative potency and the time course of the effect of 10 mg/kg CP dose were similar in rats, rabbits and dogs. A possible advantage of the CP over HEP is the more prolonged in vivo anticoagulant activity.

DECREASED PHOSPHORYLATION OF NUCLEAR PROTEINS IN TUMOR CELLS AFTER TREATMENT WITH ANTICANCER DIBROMO-DULCITOL (DBD)

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DBD is a widely used anticancer agent which has a considerable effect on DNA and chromatin proteins. The phosphorylation of chromatin proteins is generally higher in rapidly proliferating cells, therefore the phosphorylation of nuclear proteins was examined in tumor cells after DBD treatment. Rats bearing Novikoff hepatoma ascites tumor cells were treated with DBD in a dose of half of the LD_{50}. Three or 17 hrs later cells were collected, washed and incubated with radioactive inorganic phosphate for 2 hours. Nuclear and chromatin proteins were isolated and separated by one and two-dimensional gel gelectrophoresis. It was found that the phosphorylation decreased considerably both in histones and especially in non-histone proteins (by 50%) as compared with proteins from untreated tumor cells. Certain non-histone proteins showed specific differences in phosphorylation after DBD treatment: (1) A protein of 15,000 D which was heavily phosphorylated in untreated cells did not exhibit phosphorylation after treatment. (2) The protein fraction of 30-32,000 D (pH 6.8-7) was phosphorylated intensively in spite of the overall decrease of phosphorylation.

The decreasing of phosphorylation of nuclear proteins in tumor cells after DBD treatment indicates a trend toward normal regulation.

PHARMACOLOGICALLY ACTIVE cAMP PHOSPHODIESTERASE INHIBITORS

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Inhibition of cAMP phosphodiesterase (PDE) by some pharmacologically active (smooth muscle relaxant) pyrido-pyrimidines and isochinoline derivatives (synthesized in Chinoin) were studied. This enzyme plays an important role in the regulation of the intracellular cAMP level and is involved in the modification of several physiological processes (smooth muscle relaxation, platelet aggregation etc.).

cAMP PDE was isolated from bovine heart, rabbit lung and human platelet, and the enzymic activity was measured at different drug concentrations. I_{50} values (μM) were calculated and compared to well-known cAMP PDE inhibitors (papaverine and teophyllin).

Some of these new, potential drugs showed effective inhibition of the cAMP PDE enzymes, furthermore in some cases organ specificity was observed. On the basis of these results one can assume that in the mechanism of action of these drugs one of the targets is the cAMP PDE enzyme. The relationship of the chemical structure and the inhibitor potency of these drugs will be discussed.

INTERACTION BETWEEN PENICILLIN-BINDING PROTEINS AND BETA-LACTAM COMPOUNDS

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The antibiotic action of beta-lactam compounds is exerted by their inhibitory effect on some of the membrane enzymes (socalled PBPs) involved in the bacterial cell wall synthesis. The basic condition of the inhibitory effect is the formation of a covalent bond between the beta--lactam molecule and the target enzyme. This binding can be studied in vitro in the isolated cytoplasmic membrane of bacteria.

Membranes of Gram$^+$ Streptococcus faecalis ATCC 9790 and Gram$^-$ E.coli HB 101 bacteria were isolated, and the binding of some new derivatives of semi-synthetic cephalosporines to the PBPs was investigated.

The affinity of the cephalosporines can be determined on the basis of the competition between the cephalosporin and ^{3}H-penicillin G. Separation of the membrane proteins and the detection of PBPs were performed by SDS-gel-electrophoresis and fluorography.

In the S.faecalis strain a correlation exists between the inhibition of PBP1, PBP2 and the MIC (minimal inhibitory concentration) values. In the Gram$^-$ bacterium - where the outer membrane represents a permeability barrier - some derivatives give high MIC values, however, they have high affinity to the PBPs in vitro. In vivo the penetration of these compounds through the outer membrane as well as the interaction of the target enzymes are - supposedly - strongly inhibited.

IMPROVEMENT OF THE SOLUBILITY OF STEROIDS BY FORMATION OF CYCLODEXTRIN INCLUSION COMPLEX

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Poor water solubility and low dissolution rate of steroids often cause difficulties during industrial production or medicinal application. Some of these can be avoided without modifying the chemical structure of molecules through cyclodextrin inclusion complex formation. Interaction of 16 different steroids with α-, β-, γ- and diMe β-cyclodextrin has been investigated. Solubility of steroids in different aqueous cyclodextrin solutions can be enhanced 2-1200 fold, as compared to that in water. Stability constants of the complexes - calculated from solubility studies - are in the range of 60-13,000 M^{-1}; for complexes of a certain steroid the values usually increase in the order $\alpha < \beta \approx \gamma <$ diMe β.

At variance with the free drugs, the solid crystalline complexes having an active ingredient content of ~10% (w/w) get wet easily, and their hydrophilicity enables rapid dissolution. After pouring complex powders into water and stirring the system, dissolution equilibria can be achieved within 1-5 minutes, while aqueous solutions of the free drugs become saturated in 40-120 minutes under similar conditons.

The efficiency of the bioconversion during production can be enhanced, and promising in vivo results indicate that the biological effect of drugs can also be improved by using different steroid--cyclodextrin complexes.

CORTISOL AND ITS CYCLODEXTRIN COMPLEXES: BEHAVIOUR IN VITRO AND IN VIVO

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The alpha-, beta- and gamma-cyclodextrin complexes of cortisol were prepared. The molar ratio of cortisol to cyclodextrins was about 1 to 2.5. The content of cortisol in all three complexes was approximately 10%, w/w. The stability constants of these complexes were different.

We examined the solubility of complexed cortisol and of free cortisol (powder) in water at 37°C. The complexed form of cortisol was dissolved much more rapidly than free cortisol, and its solubility was 4-5 times higher. The permeation of cortisol complexes through a "Nephrophan" membrane took place 2-3 times faster than the permeation of free cortisol.

Animal experiments: We used adult rats. Blood samples were taken at various times after oral administration of the complexes and cortisol itself. All plasma samples were analyzed by radioimmunoassay.

Oral administration of the alpha complex resulted in higher plasma cortisol levels than the ingestion of free cortisol. After 1 hour the plasma concentrations were two times higher, later 4-5 times higher. Similar results were obtained with the other complexes.

STABILIZATION OF PROSTACYCLIN BY β-CYCLODEXTRIN INCLUSION COMPLEX FORMATION

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The wide application of prostanoids - especially that of the prostacyclin (PGI_2), the effective antiplatelet and cytoprotective agent - is limited by their instability. To overcome this problem either new, more stable analogues are synthetized, or the endogenous substance is stabilized by cyclodextrin (CD) complexation (Szejtli et al, Hung. Pat. 179 141, 1977).

The rates of hydrolysis of PGI_2 and its methyl ester (PGI_2Me) in aqueous solution were significantly retarded by α-, β- and γ-CD. PGI_2Me--β-CD complex has the highest stability constant (Uekama et al, Chem. Pharm. Bull. 29 213, 1981). The β-CD complex of PGI_2Me was prepared from a homogenous solution by freeze-drying. The active ingredient of the product is 3%. The complex formation was assessed in solid state by X-ray diffractometry and thermal analysis (TG, DSC, EGA curves). The freeze--dryed powder can be stored at $0^{\circ}C$ for 1,5 years, while the free drug only for 4 months according to the accelerated investigations of stability. The biological effectiveness of the complex agrees well with that of the active ingredient. The stability of the complex compared to the free drug was about 5-8 fold in the antiaggregation test according to Born. After oral administration the cytoprotective effect of the free and complexed forms of the drug against the indomethacin and stress induced ulcer in rats were similar in their magnitude, however a more prolonged effect of the complex was detected in this case, too (Balint, Internal report, 1983).

ABSORPTION AND EXCRETION OF TABLET-DISINTEGRATING β-CYCLODEXTRIN POLYMER IN RAT AFTER PER OS ADMINISTRATION

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β-Cyclodextrin is a ring-shaped molecule consisting of 7 glucopyranose units. Having an inclusion complex forming ability, β-cyclodextrin can be widely used in the drug and food industry. Recently, the introduction of cyclodextrin polymers have opened a new perspective of the application of cyclodextrins. One of the β-cyclodextrin polymers, crosslinked by epichlorohydrin can be used as a tablet-disintegrating agent due to its rapid and high swelling capacity. In the respect of pharmaceutical application, it is very important to know whether this polymer is absorbed from the gastrointestinal tract or not. The aim of this study was to investigate the absorption of β-cyclodextrin polymer in rat after per os administration. ^{14}C-labeling was performed by the use of 2-^{14}C-epichlorohydrin for the polymerization process. Radioactivity of blood, urine, feces, gastrointestinal tract and exhaled CO_2 was measured by liquid scintillation method. No radioactivity could be detected in blood in 24 hours. Radioactivity of urine and exhaled CO_2 together did not exceed the 0.11% of the total administered radioactivity. Practically all administered radioactivity (98%) was found in the large intestine and the feces. According to these results, it could be concluded that the β-cyclodextrin polymer was not absorbed from the gastrointestinal tract, therefore probably it has no toxic effect and can be safely applied in the drug industry.

DISTRIBUTION OF RADIOLABELED SALICYLIC ACID IN RATS

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Distribution of salicylic acid - an antiinflammatory prostaglandin antagonist drug - was examined in adult female, infant and pregnant rats and in their fetuses as well as in lactating rats and in their infants. After oral administration of tritiated salicylic acid its concentration was measured in the serum, urine, vegetative and endocrine organs and in the uterus of pregnant rats and rats with implanted intrauterine device. The results were compared with the distribution of two natural compounds, tritiated leucine and thymidine administered i.v. The activity of the homogenates of different organs was measured by liquid scintillation method. Salicylic acid shows rapid absorption. Its concentration in the serum decreases continuously at variance with that of thymidine and leucine, which rapidly fall, but the serum level of thymidine stagnates two hours after administration. The excretion and the decrease of radioactivity in the organs are the most rapid in the case of salicylic acid. The ratio of the specific radioactivity of the organs and the serum (relative specific activity) decreases in the case thymidine, and it increases in the case of leucine and salicylic acid. After salicylic acid administration the specific radioactivity was high in the liver, kidney, pituitary and uterus. The intrauterine device significantly increased the specific radioactivity of the implanted horn of the uterus. According to our measurements about 4-7 per cent of salicylic acid can reach the fetuses through the placenta. About 0.7 per cent of the slicylic acid given to the lactating mothers is transferred to their infants by the milk.

A NEW CHROMATOGRAPHIC DETERMINATION OF CYCLOSPORIN A

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Cyclosporin A is an extremly effective immunosupressant, employed at present in organ and bone marrow transplantations. The side effect that needs to be watched most is the dose dependent and reversible nephrotoxicity. Thus a reliable method to determine blood levels of CyA is obviously necessary. We have developed a new procedure and used it in 2 cases after transplantation.

Principle: CyA is hydrolysed by HCl and a dinitroproduct of the hydrolysate is determined by HPLC at room temperature.

Procedure: 5 ml of the sample (plasma) was mixed with 5 ml of dist. water and 40 ml of acetonitrile. The mixture was boiled for 1-2 min then cooled and centrifuged at 3000 g for 10 min. An aliquot of 45 ml was transferred to a 50 ml test tube; and after drying, 1 ml of 6 N HCl was added. The time of hydrolysis was 120 min. The hydrolysed sample was dried at room temperature and solved in 0.1 ml TRIS in water (cont. 20 g/l) + + 0.1 ml dist. water + 0.3 ml acetonitrile + 0.2 ml of 1-fluoro-2,4--dinitrobenzene in acetonitrile (cont. 170 g/l). This sample was incubated for 60 min at 70°C then cooled and an aliquot of 25 µl was injected into the column. The valin component of the hydrolysed CyA was determined. (Capacity ratio/k'/:2.70; line of calibration:Y = 0.07 X;). The sensitivity was: 20 µg/l. Range : 20 - 2000 µg/l.

Instrument: Liquochrom-2010 (Labor MIM, Hungary). The mobil phase: : 300 ml of water, 700 ml of acetonitrile and 1 ml acetic acid. The flow-rate:0,5 ml/min. Column: Lichrosorb RP-18 (250 x 4.6 mm I.D., 7 µm)--Merck. Ultraviolet detection at 365 nm.

Employing the above described method we monitored serum levels of CyA in two cases, one after kidney- and another, after bone marrow transplantation (at the I.Surgical Dept. of Semmelweis Univ. and at the National Institute of Hematology). The serum levels of CyA in both cases varied within the therapeutic range.

INHIBITION OF VERATRIDINE-INDUCED DEPOLARIZATION OF SYNAPTOSOMES BY VERAPAMIL

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^{86}Rb distribution of rat brain synaptosomes was measured and the approximate value of membrane potential was calculated by Nernst equation. Veratridine decreased $Rb_{in}:Rb_{out}$ ratio in a concentration-dependent manner indicating depolarization of the membrane. Verapamil, regarded as a selectiv inhibitor of Ca-channels, at 2-100 μM concentrations inhibited veratridine-induced depolarization. The effect seemed to be specific since verapamil itself in these concentrations did not cause depolarization and it had no effect on depolarization induced by high K^+ (25 mM) or ouabain (50 μM) either. Verapamil inhibited ^{45}Ca uptake enhanced by veratridine, however, in concentrations where depolarization was also prevented. It is suggested that in addition to its well-known effect on Ca-channels verapamil might also interfere with Na-channels of synaptosomes.

EFFECTS OF IN VIVO GABA TREATMENT ON THE ACTIVITY AND MOLECULAR FORMS OF ACETYLCHOLINESTERASE IN THE RAT SUPERIOR CERVICAL GANGLIA

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In addition to acting as an inhibitory neurotransmitter, it has been suggested that GABA may act as a trophic factor during development (Volff, Behav. Devel., 164, 1981). When the superior cervical ganglion (SCG) was exposed to GABA, special membrane thickenings were formed at the extrasynaptic dendritic surface of the ganglion cells (Wolff et al, J. Neurocytol. 8 549, 1979). It has been suggested that these free postsynaptic membrane thickenings (F-post) might become functional if the n. hypoglossus is implanted into the SCG after GABA treatment (Dames et al, Nature in press).

It has also long been known that acetylcholinesterase (AChE: EC 3.1.1.7) is one of the components of the postsynaptic membrane. The main goal of our experiments was therefore to investigate the effect of (a) GABA treatment and (b) GABA treatment plus n. hypoglossus implantation on the AChE activity and the distribution of its molecular forms.

One day following in vivo GABA administration, the AChE activity in the SCG was decreased to 60-70% and remained near this level for a week. During this period the distribution of the molecular forms (4S, 10S and 16S) did not change as compared to the control values. 1.5-2 months following GABA treatment plus n. hypoglossus implantation there was a significant increase (40%) in the enzyme activity, and among the molecular forms the amount of 16S was doubled.

These results may explain the maturation of the F-post synaptic thickenings to become functionally active synaptic sites. This hypothesis is supported by the fact that the 16S AChE form was localized mainly at the synaptic junction.

GLUTAMIC ACID DECARBOXYLASE ACTIVITY IN PERIPHERAL TISSUES OF MAMMALS

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Glutamic acid decarboxylase (GAD) is primarily responsible for the biosynthesis of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) in the brain. The presence of GABA and GAD in certain peripheral tissues has also been reported. The methods used for GAD activity measurement are based on the detection of GABA or CO_2 formation from glutamic acid. Recent studies have revealed that in certain peripheral tissues the CO_2 trapping procedure may lead to overestimation of GAD activity due to indirect formation of CO_2 from glutamic acid. In the present study, the stochiometry of GABA and CO_2 production was examined in various peripheral tissues of the rat, rabbit and man, in order to obtain appropriate information regarding the local formation rates of GABA. In most tissues examined, significant CO_2 production could be observed, while the rate of GABA formation was much lower, even if detectable. Stochiometric GABA and CO_2 synthesis could be found in the rat brain and oviduct. The GAD activity in these two organs showed similar time course, temperature dependence, the same pH-optimum (6.8), cofactor requirement and kinetic constant (K_m about 1.5 mM). The responsiveness of the two enzymes to various inhibitors was also consistent. The above findings indicate that (1) GABA is synthetized locally in a series of peripheral mammalian tissues (2). The CO_2 trapping method is not adequate for GAD activity measurement in most peripheral tissues (3). The rather similar biochemical characteristics of brain and oviduct GAD suggest the presence of closely related enzymes in the reproductive organ and in the nervous tissue.

INVESTIGATION OF PRESYNAPTIC EFFECTS OF KAINIC ACID IN THE HIPPOCAMPUS OF RAT

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Kainic acid (KA), a neurotoxic analogue of glutamic acid (Glu), destroys and degenerates cell bodies in the CNS. The mechanism of this effect is not yet understood. In this study our aim was to gain more information on the effect of KA on the cholinergic system.

The experiment was carried out on hippocampus tissue slices (350 μm). The released ACh induced by 0.5, 1.0, and 10 mmol/l KA was measured by a modification of the method described by Somogyi and Szerb (1972). After KA treatment the tissue slices were re-stimulated with 50 mmol/l KCl.

The results show that the effects on ACh release of 0.5 and 1.0 mmol/l KA are similar. The amount of ACh increased in a dose-dependent manner between 1.0 and 10 mmol/l KA treatment. 0.5 and 1.0 mmol/l Glu did not induce any ACh release, but 10 mmol/l had an effect similar to that of 1.0 mmol/l KA.

From these results it is inferred that KA influences not only the postsynaptic dendrites and perikarya, but also the cholinergic septo--hippocampal axon terminals. It is suggested that KA receptors may be present on the presynaptic axon terminals.

EFFECTS OF IN VIVO 4-(1-NAPHTHYLVINYL)-PYRIDINE (NVP) ON THE SUPPLY OF CHOLINE ACETYLTRANSFERASE WITH ITS SUBSTRATES

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One of the basic questions of the biochemistry of the cholinergic system is the regulation of ACh synthesis. It has been suggested that the rate of choline (re-)uptake, the product inhibition of CAT activity or the mass action law may play an important role in the regulation.

In this study our aim was to elucidate whether NVP (a well-known CAT inhibitor) could also influence the high-affinity choline uptake (HACU), endogenous AcCoA level, free choline content and Ach level in different parts of the brain.

Our results show that NVP (200 mg/kg) significantly inhibited the in vivo activity of CAT and the HACU. The AcCoA content of the tissues did not differ from the control value. After NVP treatment the ACh contents of the hippocampus, cortex and cerebellum were reduced by 50 per cent. If the NVP administration was followed by swimming stress for 20 min, the ACh level was reduced almost to zero in all regions. The free choline content of all brain parts was elevated significantly, however. This increased choline level is presumably due to the accumulation of choline in the extracellular space.

THE EFFECTS OF AF64A IN THE CENTRAL NERVOUS SYSTEM: BIOCHEMICAL AND MORPHOLOGICAL INVESTIGATIONS

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Earlier results have shown that i.c.v. administration of a cholinotoxin, AF64A, selectively affects the elements of the ACh system. It reduces the activity of choline acetyl-transferase (CAT: EC 2.3.1.6), the high-affinity choline uptake (HACU) and the level of acetylcholine (ACh).

In this study our main goal was to gain more information about the i.c.v. and i.c. effects of AF64A on the morphology and cholinergic system of the septum, hippocampus and striatum. Rats of the CFY strain were used. AF64A was injected either i.c.v. (5, 8, 20, 40 and 65 nmol) or into the striatum (0.5 and 8 nmol). After different time intervals the activities of CAT and acetylcholinesterase (AChE: EC 3.1.1.7) were measured and the ultrastructure of the neurons was investigated. From these results it is inferred that the cholinotoxin AF64A reduces the activity of CAT first (presynaptic effect) and after some days the activity of AChE decreases as well (post-synaptic effect). During this process some of the axon terminals degenerate.

THE EFFECT OF CHRONIC l-DEPRENYL TREATMENT ON THE UPTAKE OF BIOGENIC AMINES AND MAO IN RAT STRIATUM

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The pharmacological effect of l-deprenyl (l-D), a selective and irreversible inhibitor of MAO-B, can not be explained by its enzyme--blocking property alone. We have examined the effect of l-D pretreatment (0.25 mg/kg, s.c. for 1, 2, 14 and 20 weeks) on the uptake of ^{3}H--noradrenaline (^{3}H-NA), ^{3}H-dopamine (^{3}H-DA) and ^{3}H-serotonin (^{3}H-5HT) in crude synaptosomal preparation by a modified method of Snyder and Coyle (J. Pharm. exp. Ther. 165 78, 1969). The selectivity of MAO-inhibition was studied by using the method of Otsuka and Kobayashi (Biochem. Pharm. 13 995, 1964). Data of in vitro experiments show that l-D inhibits ^{3}H-NA and ^{3}H-DA uptake but does not influence the uptake of ^{3}H-5HT (IC_{50} values 3.6×10^{-5}; 8.7×10^{-5}; $> 10^{-3}$ M, respectively). During the chronic treatment for 14 and 20 weeks the selective MAO-B blocking pattern remains unaltered but a remarkable change in the uptake-inhibition develops gradually. In rats treated for 20 weeks ^{3}H-NA uptake becomes strongly inhibited (46.3%), ^{3}H-DA uptake seems to be unchanged (97.9%) and ^{3}H-5HT uptake shows a strong facilitation (217.1%). We conclude that the permanent, selective MAO-inhibition may cause changes in neurotransmission.

DIFFERENTIAL EFFECTS OF l- and d-DEPRENYL (D) IN RAT BRAIN

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Effects of l- and d-D on MAO activity of rat brain, on striatal levels of dopamine (DA) and 3,4-dihydroxy-phenylacetic acid (DOPAC: a metabolite of DA) and on striatal uptake of DA were determined. MAO activity was measured radiochemically by the method of Otsuka and Kobayashi (Biochem. Pharmac. 13 995, 1964). DA uptake was determined as described by Snyder and Coyle (J. Pharmacol. Exp. Ther. 165 78, 1969). DA and DOPAC levels were measured by using HPLC-EC (Sperk, J. Neurochem. 38 840, 1982). Drugs (2 and 8 mg/kg) were given i.p. and their effects determined 4 and 48 hours after treatment. Both l- and d-D were weak inhibitors of DA uptake (IC_{50} values: 1×10^{-4} and 2.3×10^{-4} M, respectively). The higher dose of d-D reduced MAO-B activity by 54% and did not affect the A form of the enzyme, while l-D inhibited MAO-B by 91% and MAO-A by 57% (MAO activity was determined 4 hours after the administration of D). The inhibition pattern was similar when MAO-activity was measured 48 hrs after D-treatment. DOPAC levels were decreased by both forms of D to a similar extent (26 to 33%) 4 hrs after the injection of D. The effect of d-D disappeared completely while the effect of l-D was only slightly reduced 48 hrs after drug administration.

Our data suggest that l-D acts as a selective and irreversible MAO--inhibitor whereas d-D blocks MAO reversibly.

cAMP MICROCOMPARTMENTATION AND PROTEIN PHOSPHORYLATION IN WILD TYPE AND MEMORY-MUTANT DUNCE STRAINS OF DROSOPHILA MELANOGASTER

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The dunce mutants of D. melanogaster are deficient in memory and have elevated cAMP levels owing to the diminished activity of a cAMP--phosphodiesterase isoenzyme (PDE-II). Microcompartmentation via binding sites of cAMP was compared in wild Canton-S and dunce-M11 flies. Total cAMP content was 1.6 $\pm$ 0.3 and 10.5 $\pm$ 1.7 pmol/mg fly resp., as determined by RIA. Kinetic analysis of cAMP decay in homogenates by endogenous PDEs revealed at least two cAMP pools: free cAMP and cAMP bound to the R-subunit of cAMP-dependent protein kinase (cAMP-PK). The R.cAMP level in dunce-M11 is about 2-3 times as high as in Canton-S. Phosphorylase b was purified from Canton-S flies and characterized. The phosphorylase a level was 20% in Canton-S and 34% in dunce-M11, indicating that high cAMP level exerts slight, if any, effect on the state of activation of phosphorylase.

Protein phosphorylation in vitro with (γ^{32}P) ATP in fly head homogenates produced one major labelled band (app. $M_r \approx 53000$) which probably is the R-subunit of cAMP-PK. cAMP, while needed for labelling this band, increased dephosphorylation at higher concentrations (~10 μM). It is hypothesized that enhanced dephosphorylation underlies the memory deficiency of dunce flies.

TUMORIGENICITY OF GLUCOCORTICOID SENSITIVE AND RESISTANT, DIFFERENTIATED AND DEDIFFERENTIATED HEPATOMA CELLS

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The growth of cell clones derived from the transplantable hepatocellular Reuber carcinoma is inhibited by glucocorticoids. It has been possible to isolate glucocorticoid (dexamethasone: dex) resistant variants from the originally growth sensitive hepatoma cell clone Faza 967. All the isolated dex-resistant variants were shown to contain functioning glucocorticoid receptors. The tumorigenicity of cell clones of identical histogenetic origin, differing in dexamethasone sensitivity and state of differentiation, was examined. Neither the degree of differentiation nor glucocorticoid resistance influenced the tumor forming capacity of Reuber rat hepatoma clones. However, the tumorigenicity of those, independently isolated, resistant clones which were maintained in vitro continuously in the presence of high concentration of dexamethasone for more than a year decreased considerably. The fact that not only the differentiated but also the partially dedifferentiated and the dedifferentiated hepatoma cells grew in form of tumors in nude mice made possible to examine whether reexpression of the extinguished, liver-specific functions may occur in the tumors. Reexpression of some liver-specific functions of the tumor cell lines derived from a partially dedifferentiated, dexamethasone resistant clone was found. Our observations indicate that in vivo tumor formation induces the differentiation rather than selects for differentiated cells.

IN VITRO AND INTRACELLULAR THYMIDINE PHOSPHORYLATION IN EXPERIMENTAL TUMOR CELLS

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Thymidine kinase activity was measured in vitro in the cytosol of Ehrlich and L1210 in vivo growing ascites tumors during the whole growth period and in the cytosol of the following tumor lines, growing in cell culture: P388 parent, TK and HPRT deficient mutants and hybrid of the two mutant lines. The enzyme activities showed only a limited correlation with cell kinetic parameters suggesting that the in vitro enzyme activities do not reflect the intracellular circumstances. To get information on the intracellular situation, experiments were carried out to calculate the intracellular enzyme activities. Cells were incubated in the presence of different concentrations of labeled thymidine. The phosphorylated and non phosphorylated thymidine content of the acid soluble supernatant was separated chromatographically. The radioactivity of the fractions and of the acid precipitate was measured. The intracellular enzyme activities calculated on this basis were only about 1% of those measured in the cytosol but showed a better correlation with cell kinetic parameters and they reflect better the intracellular situation than those measured in the cytosol in vitro.

LOCALIZATION OF DNA REPLICATION AND REPAIR AFTER 3-MC TREATMENT IN RAT

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Due to increasing interest in chemical carcinogenesis 3-methyl-cholantrene (3-MC), one of the polycyclic aromatic hydrocarbons, has been studied. 3-MC causes a long-patch repair (nucleotide excision repair) in the cells.

Replication and repair (unscheduled) synthesis of DNA in lung and liver (as control) were studied in our experiments. CFY rats were treated with a single dose of 3-MC intratracheally and i.p. (50 mg/kg b.w.). The animals were killed 1, 4 and 28 days after the treatment. (^{3}H)-thymidine was added 1 hour before killing in a dose of 0.75 mCi/kg b.w. and lung and liver samples were prepared for light microscopic autoradiography.

From our investigations the following conclusions can be drawn: 1/ Replication of DNA in the lung increased very slowly during the investigated periods. 2/ Repair of DNA in the lung increased quickly and the maximal activity was observed on the 4th day after 3-MC treatment. 3/ There was a minimal effect on liver after intratracheal treatment of 3-MC. 4/ Replication and repair occurred mainly subepithelially in the lung.

CHANGE OF SOME PARAMETERS DURING DNA REPAIR INDUCTION

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Some parameters of induced DNA repair of mouse spleen lymphocytes were examined after in vivo treatment with methyl-methanesulfonate (1 x 100 mg/kg), acetylaminofluorene (4 x 5 mg/kg), and γ-irradiation (Co^{60}) (1 Gray). The time-course of the rate of DNA repair synthesis (reaching a maximum and then returning to the control level) was different after treatment with the various inducers. The activity of ADP--ribosyltransferase changed parallel with DNA repair synthesis. The UV--dose necessary to produce half-maximum rate of DNA repair synthesis depended on the inducer as well as on the time of induction. Endonuclease and exonuclease activity - two significant factors of the DNA repair - were also measured. These results suggest that the rate limiting step of the complex enzymatic process changes with the type of inducer and the time of induction.

THE ROLE OF ACTIVATING AND DETOXIFYING ENZYMES IN BINDING OF REACTIVE BENZO(a)PYRENE METABOLITES TO DNA IN MICROSOME-MEDIATED SYSTEMS

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In chemical carcinogenesis the binding of reactive electrophilic metabolites to DNA is considered essential to the initiation of the carcinogenic process. Formation of reactive and nonreactive metabolites occurs in very complex enzyme interactions. Combined effects of cytochrome P-450 dependent monooxygenase, epoxide hydrolase, UDP--glucuronyltransferase and glutathione S-transferase were measured on total binding of benzo(a)pyrene (BP) metabolites to calf thymus DNA as well as to DNA of Chinese hamster overy cells in the presence of liver microsomal and cytosol fractions from 3-methylcholanthrene treated rats. UDP-glucuronic acid (UDPGA) alone, or in combination with glutathione (GSH) and/or 1,2-epoxy-3,3,3-trichloropropane (TCPO, an epoxide hydrolase inhibitor) increased the binding by 100%. In the cellular model system both UDPGA and GSH decreased the amount of DNA adducts, either alone or in combinations with TCPO, and a 50% decrease was obtained in the simultaneous presence of the two conjugating agents. The negative correlation between the results of the two model system may be due to the barrier function of cell the membrane towards hydrophilic conjugates. The different binding patterns of the main BP metabolites can be due to the selective trapping of them by cellular macromolecules other than DNA.

THE EFFECT OF TUMOR-PROMOTER PHORBOL-ESTER ON THE METABOLISM OF CARBAMOYL-PHOSPHATE AND ORNITHINE IN MICE

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Mammalian cells can use carbamoyl-phosphate and ornithine in many different ways, i.e. for polyamine, arginine (urea) and pyrimidine synthesis. In resting tissues the synthesis of amino acids predominates, whereas in proliferating tissues the polyamine and pyrimidine synthesis becomes preponderant.

We investigated the metabolism of carbamoyl-phosphate and ornithine in mice after skin painting with tumor-promoter 12-O-tetradecanoylphorbol--13-acetate (TPA). Five to twenty hours after treatment the activity of glutamine-dependent carbamoyl-phosphate synthetase, aspartate carbamoyltransferase, dihydroorotase and ornithine decarboxylase was enhanced, that of ornithine carbamoyl-transferase and ornithine aminotransferase was diminished, whereas the activity of the ammonia--dependent carbamoyl-phosphate synthetase remained constant.

Our results suggest that TPA may induce temporary changes characteristic of tissue proliferation, i.e. the extramitochondrial polyamine and pyrimidine synthesis is increased on account of intramitochondrial arginine (urea) production.

PLACENTAL PROTEINS AS POTENTIAL TUMOR MARKERS

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Three placental proteins, placenta protein 10 (PP_{10}), placental protein 12 (PP_{12}) and pregnancy-specific beta-1-glycoprotein (SP_1) were studied in sera of patients with different malignant tumors. The aim of this work was to develop methods for the measurement of these proteins and to establish their clinical significance. We investigated 50 healthy and 198 pathological sera using radioimmunoassay or enzyme-linked immunoassay methods.

PP_{10} could be detected in 70% of nonpregnant women's sera and 52% of male sera (1.81 and 2.02 ng/ml). PP_{12} was found in 94% of women and 52% of men (26.2 and 23.6 ng/ml). SP_1 was demonstrable only in trace amounts in 10% of healthy controls. The serum levels of these proteins during pregnancy and in cases with trophoblastic diseases were examined and published earlier.

Elevated levels of PP_{10} were found in patients with breast and vesical cancer (3.24 and 2.95 ng/ml). Raised PP_{12} concentrations can be measured especially in patients with cancer of the prostate (86.8 ng/ml). SP_1 showed higher values in 12.8% of 94 patients suffering from primary pulmonary cancer (5.1 ng/ml). There were no significant correlations with other tumor markers as alpha-foetoprotein or pregnancy-associated alpha--2-glycoprotein. Placental proteins can be regarded as tumor associated proteins and they can provide useful informations especially in the individual follow-up investigations.

PRESENCE OF MYELIN BASIC PROTEIN IN THE CEREBROSPINAL FLUID AND SERUM OF PATIENTS WITH MULTIPLE SCLEROSIS

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Demyelination, or the loss of the myelin, characterizes a number of neurologic diseases. One of these is multiple sclerosis. Cohen et al. (New England J. Med. 295 1455-1457, 1976) suggested that an assay for myelin or one of its components might have diagnostic value. The myelin basic protein (MBP) is the most thoroughly characterized myelin component. Its polipeptide chain consists of 170 amino acid residues.

Cerebrospinal fluid and serum from multiple sclerosis patients were tested by affinity chromatography using monoclonal antibodies. Two types of monoclonal antibodies were conjugated to Sepharose 4B matrix and used for binding MBP. One of the two types binds MBP at the N terminal region between residues 1 and 117, the other binds it at the C terminal region between residues 117 and 170. Detection of MBP and/or fragments of MBP in the eluted solutions were made by using SDS gelelectrophoresis combined with a silver-staining method.

We found that the MBP content of the cerebrospinal fluid is very high (up to 100 ng) in pathological cases of active demyelination (multiple sclerosis) in contrast to the control population (1-2 ng). The SDS gelelectrophoresis experiments show that fragments of MBP are also present in the cerebrospinal fluid and serum of patients.

ORNITHINE DECARBOXYLASE ACTIVITY IN THE LUNGS OF MICE AFTER LOBECTOMY

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Ornithine decarboxylase (ODC; EC 4.1.1.17) is the first, rate--limiting key-enzyme of polyamine biosynthesis. After partial hepatectomy the ODC activity shows biphasic changes. To find out whether this behavior is specific for the liver or characteristic of regenerating tissues in general, we measured the ODC activity in the lung of female BALB/c mice according to Russell and Snyder (PNAS 60 1420, 1968), after removing the left upper lung-lobe (lobectomy).

It was found that a compensatory growth begins in the lungs after lobectomy. The ODC activity was enhanced five-fold after 4 h and showed a second increase between the 12th and 48th h. Each lobe exhibited different ODC activity, and it was the highest in the left lower lobe. When the mice were treated with 8 mg/kg Actinomycine D or with 6 mg/kg cycloheximide before lobectomy, the induction of ODC did not occur. This suggests that the induction of ODC is a result of de novo protein synthesis.

Since we wanted to find out whether the regeneration is elicited by local (mechanical) or by humoral (chalones) factors, the left lower lobe of mice was transplanted under the renal capsule of sister mice. An increased ODC activity was observed after lobectomy both in the own and in the transplanted lobe. The ODC activity of the own lobe was twice as high as that of the transplanted one. The results indicate that the compensatory growth can be elicited by local and humoral factors, as well.

PEROXIDATIVE CHANGES IN THE EARLY PHASE OF EXPERIMENTAL SILICOSIS

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In our earlier investigations it was established, that the standard dust (DQ-12), containing high concentrations of quartz exerts an oxidative effect in the lung in vivo (T. Zsoldos et al, Exp. Pathol. 23 73-77, 1983). According to our assumption, the quartz induces enzymes which generate oxygen free radicals, which cause the lipid peroxidative type damage of cellular membranes. In order to analyse the effect of SiO_2 in the early phase of silicosis, we have carried out comparative investigations with pure coal. The particle diameter of the coal dust was the same as that of DQ-12. Wistar albino rats were exposed to dust intratracheally, for 3-6-12-21 days. It was observed that the malondialdehyde (MDA) concentration was higher by 140%, the superoxide dismutase (SOD) activity by 110%, and the reduced gluthatione (GSH) by cc. 250% in the case of DQ-12 administration, than after using pure coal. In parallel with this the tissue redox potential was also higher following quartz-containing dust exposition. The intracellular localization of endogenous peroxidases was also studied. There was a significant increase in the number of diaminobenzidine (DAB) positive granules in the alveolar macrophages. These are partly peroxisomes, partly the elements of the phagosomes containing the phagocyted bodies. The activity in the second type pneumocytes is connected with the GER.

STUDY OF FACTORS PLAYING A ROLE IN THE POSSIBLE CELLULAR REDOXPOTENTIAL ALTERATION IN PATIENTS WITH PORPHYRIA CUTANEA TARDA

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Porphyria cutanea tarda (PCT) accompanied by uroporphyrin-uria and characteristic cutaneous symptoms is the most frequent of the porphyrin metabolic diseases. Besides other factors, the alteration of the cellular redoxpotential is possibly playing a role in the development of the disease.

During earlier investigations we demonstrated that the activity of the erythrocyte 5-aminolaevulinic acid dehydratase (EC 4.2.1.24.) is decreased in patients with PCT; this enzyme requires a -SH activator, and is thus very sensitive to redox changes.

This prompted us to examine some components of the erythrocyte redox system, the whole blood reduced glutathione (GSH) content, and the activities of the erythrocyte glucose-6-phosphate dehydrogenase (G6PD) (EC 1.1.1.49.) and glutathione reductase (GR) (EC 1.6.4.2.) in our patients with PCT.

The whole blood GSH level for the PCT patients (0.84 mmole/l) was significantly lower than that for the control group (1.13 mmole/l). A similar decrease was observed in the erythrocyte G6PD activity (26.9 U for the PCT group and 43.1 U for the control group). The activity of erythrocyte GR was essentially the same in the two groups (22.0 U and 20.1 U).

The results show an oxidation shift in the redox state of the erythrocytes in the PCT patients.

METAL (Fe, Zn, Cu) CONCENTRATIONS IN THE HAIR OF PATIENTS WITH PORPHYRIA CUTANEA TARDA

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Urinary porphyrin levels and concentrations of Fe, Zn, Cu (analyzed by atomic absorption spectrophotometry) in serum and in the hair of 38 patients with porphyria cutanea tarda (PCT) and 20 healthy controls were studied.

Both in serum and in the hair, the mean concentrations of Fe in PCT were significantly higher than in the controls (serum Fe: 26.1 $\pm$ 9.9 and 17.99 $\pm$ 5.97 μmole/l; Fe in the hair: 0.34 $\pm$ 0.42 and 0.22 $\pm$ 0.08 μmole/g, resp.). Cu concentrations in PCT were higher than the control values but the difference was significant only in case of the hair (serum Cu: 24.6 $\pm$ 10.9 and 21.9 $\pm$ 3.9 μmole/l; Cu in the hair: 0.21 $\pm$ 0.18 and 0.14 $\pm$ 0.03 μmole/g, resp.). Both in serum and in the hair, Zn concentrations in PCT were lower than the control values but the difference was significant only in serum (serum Zn: 11.8 $\pm$ 2.0 and 13.3 $\pm$ 2.7 μmole/l; Zn in the hair: 2.40 $\pm$ 1.83 and 2.52 $\pm$ 0.98 μmole/g, resp.).

In our examination, concentrations of these metals both in serum and in the hair have no correlation either with urinary total porphyrin excretion or with the uroporphyrin fraction (the latter is characteristic for PCT).

Thus, at variance with some literary data, the levels of Fe and Cu in the organism do not play a direct role in the pathogenesis of PCT. The relationship of Zn concentration and the decreased activity of ALA--dehydratase is discussed.

A MODERN PROCEDURE FOR THE DIAGNOSIS OF DIFFERENT PORPHYRIAS

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The determination of porphyrins in the urine is important in the diagnosis and classification of the porphyrias.

The urinary porphyrins were adsorbed on talc, and then converted into their methyl esters by treatment of the talc with CH_3OH - conc. H_2SO_4 (19:1, v/v). The porphyrin esters were extracted with $CHCl_3$ and were evaporated to dryness. The dry porphyrin esters were hydrolyzed in 6 N HCl. For the isolation of porphyrin acids by reversed-phase hplc a Liquochrom-307 (Labor MIM, Hungary) apparatus was used with a Hitachi MPF-4 fluorescence spectrophotometer detector (Exc. = 406 nm, Em. = 619 nm). The separation was carried out on a column (25 cm x 4.6 mm) packed with Chromsil C_{18} (Labor MIM, Hungary). The solvents for the linear gradient elution were acetonitrile (15% v/v) in 1.0 M CH_3COONH_4 buffer (pH = 5.15) (solvent A) and acetonitrile (50% v/v) in 1.0 M CH_3COONH_4 buffer (pH = 5.15) (solvent B). The flow rate was maintained at 48 ml/hour throughout. The solvent volumes were 100 ml, respectively. The capacity ratios (k') of uroporphyrin I, - III, heptacarboxylporphyrin and coproporphyrin I, - III isomers were 5.3; 5.8; 8.4; 15.9 and 16.9, respectively. The concentrations of porphyrins were calculated by the aid of diluted standard preparations (Sigma, U.S.A.).

Porphyria cutanea tarda (PCT) was accompanied by high levels of uroporphyrin I+III (500-1900 µg/l) and heptacarboxylporphyrin (450-1000 µg/l). The single urine specimen that was from a patient with acute intermittent porphyria (AIP) also contained high amounts of uroporphyrin I (870 µg/l) and uroporphyrin III (350 µg/l), but no heptacarboxylporphyrin. In one case of congenital erythropoetic porphyria (CEP) the coproporphyrin III content (1100 µg/l) was more than that of uroporphyrin I+III (620 µg/l).

LIPID PEROXIDATION AND SCAVENGER MECHANISMS IN EXPERIMENTALLY INDUCED HEART ISCHEMIA

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Dog experiments were performed to investigate the lipid peroxidation (measured by formation of malondialdehyde, MDA), and the scavenger activity (determined by GSH and SOD contents).

The experimental groups were as follows: 1/ control dogs with intact heart. Samples were taken from the anterior and posterior wall of the left ventricle and from the right ventricular wall; 2/ dogs with acute LAD (r.desc.ant.lig.) having ischemic areas for 1, 2, 3 and 24 hours. Heart tissue was cut from the ischemic areas and from the same sites as in the control group.

Our results have shown that acute heart ischemia causes significant alterations in the lipid peroxidation and in the endogenous scavenger content. The MDA level gradually increased in the infarcted areas and its highest value can be found after 3 hours (143.5 $\pm$ 8.3%). After 24 hours the MDA content fell below normal level (78.5 $\pm$ 5.6%). SOD content in the control heart was 122 $\pm$ 15.4 U/g. In the first three hours its value decreased rapidly and at the end of 24 hours it was is found to be 45 $\pm$ 8.5 U/g. At the same time GSH is moderately reduced; after 24 hours its level was 50% of that of the normal heart tissue.

Increased peroxidation of membrane lipids always indicates the breakdown of the PUFAs in the membranes. The peroxidative damages may also alter the cellular glutathione content. Decrease of SOD activity is very important, too, bacause the dangerous effects of free radicals produced during myocardial ischemia cannot be prevented.

PATHOMECHANISM OF INFLAMMATORY PHENOMENA ASSOCIATED WITH ARTHROPATHIES

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11 parameters (WBC, rheuma-factor, superoxid-dismutase, peroxidase, catalase, proteins, acid-phosphatase, uric acid, copper, calcium, cytology) were studied in the articular fluid of 100 patients with arthropathies. The articular fluids were divided into 13 groups according to the diagnosis on admittance - modified conforming to the standard diagnostic criteria of the American Rheumatology Association. Results were then fed into a computer to carry out different statistical calculations. The examination of articular fluid facilitates differential diagnosis and the estimation of the further progress of the disease. The measurement of the activity of enzymes participating in oxygen metabolism was - as far as we know - first carried out in articular fluid by this team. A new theory has been proposed to explain the mechanism of synovitis, often concommitting articular diseases, on the basis of the activities of these enzymes and other data obtained from the measurement of different parameters as well as of literary data.

Pathological immune globulins are generated due to infection or genetical predisposition, which form immune complexes with rheuma factor, being present pathologically, too. These immune complexes are deposited in case of rheumatoid arthritis in the articular space. This - as an irritant factor - triggers the reinforcing mechanisms (complement system, kinin-way, arachidonic acid - prostaglandine cascade) as well as the release of cytokinines, free radicals anions and the changes in the concentration of acute phase proteins. According to the theory proposed herewith, both the different arthropathies and the inflammatory reactions in other organs end in a final common pathway, apart from the triggering agent and the organ involved.

ROLE OF MITOCHONDRIAL ENZYMES IN THE PATHOMECHANISM OF RHEUMATOID ARTHRITIS

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Lysosomal enzymes are important targets of connective tissue research. In this work beside the activity of the known lysosomal enzymes we followed the changes of some mitochondrial enzyme activities, too.

According to our histochemical examinations there is a significant increase of the activity of the measured enzymes (malate dehydrogenase, NADH dehydrogenase, lactate dehydrogenase, succinate dehydrogenase) in the rheumatoid nodule and in the synovial membrane. We detected an about 2-fold ubiquinone (reducible ubiquinone) content as compared to the control in the rheumatoid tissues.

The metabolism of mitochondrium, the inflammation and the destruction of tissue can be interrelated as follows. Parallel with the activity of thiosulphate sulphurtransferase the local SCN^- level was tenfold of the control in rheumatoid synovial membrane and fluid. The increased SCN^- level can activate the inactive collagenase - bound to α_2-macroglobulin - - and some other neutral proteases. On the effect of proteases an autoimmune process can be induced.

PROTECTIVE EFFECTS OF Zn^{++} AND Cu^{++} AGAINST THE DETACHMENT OF MEMBRANE-BOUND RIBOSOMES, CAUSED BY Cd^{++} IN RAT LIVER

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Cd^{++} has been reported to labilize the ribosome-membrane interaction in some cell types. In order to demonstrate this phenomenon in the liver, too, we used the gradient centrifugation methods of Blobel and Potter and found 77% of the cytoplasmic ribosomes membrane-bound and 23% of them free. Administration of 0.015, 0.025 or 0.05 mmole/kg bw. $CdCl_2$ to the rats resulted in a detachment of 5-17% of membrane-bound ribosomes within 60 min, while the total amount of ribosomes did not change. After the largest dose, the proportion of detached ribosomes reached 15% within 30 min and remained around this value until the 120th min. Equimolar (0.05 mmole/kg bw.) $ZnCl_2$, $CuSO_4$ and Pb-acetate did not cause ribosomal detachment and Zn^{++} and Cu^{++} even showed a partial protection against Cd^{++} given simultaneously for 60 min. Doubling the doses of $ZnCl_2$ and $CuCl_2$ did not elevate the degree of protection. However, 0.05 mmole/kg bw. doses of the metals given 12 h before treatment with equimolar $CdCl_2$ (for 60 min) resulted in a total prevention. X-ray fluorescence analysis of oven-dried livers showed that, given either simultaneously with, or prior to Cd^{++}, neither Zn^{++} nor Cu^{++} inhibited the uptake of Cd^{++} by the liver. Pb-acetate did not disturb Cd^{++} uptake, and it did not have a protective effect, either. Since 12 h pretreatment with 0.015 mmole/kg bw. $CdCl_2$ failed to show autoprotection against 0.05 mmole/kg bw. $CdCl_2$, metal-induced hepatic thionein might not be responsible for the protection by Zn^{++} or Cu^{++}. Incubation of rough microsomes in the presence of Cd^{++}, with or without added cytosol, showed that the detachment of ribosomes is probably not a direct action of Cd^{++} on granular endoplasmic reticulum membranes.

UTILIZATION AND NUTRITIVE VALUE OF NEWLY-BRED GRASS-SPECIES

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Digestibility of some newly bred feeder grass species (breeder: J. Janovszky, Szarvas) was investigated in vivo. Our intention was to supply detailed informations which could be used in further breeding-work and in the practical foraging.

The most important nutritive values of non-irrigated grass species was determined in each growth for three years. The digestibility was investigated with 1-2 years old wethers (30-35 kg) in metabolism-cages.

The main nutritive values tested (dry matter, crude fibre, crude protein, lysin, methionin and tryptophan) varied not only among the grass species, but also within the same species in different years and cuttings. The digestibility of nutritive components (Fibre and protein) is always higher in the first growth than in the second in all years and all species.

According to our results the revision of the foraging practice is indispensable.

A COMPARATIVE STUDY ON THE PRESERVATIVE EFFECT OF STARTER CULTURES AND ORGANIC ACIDS ON PAPILIONACEOUS SILAGE

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Preservation of fodders with great moisture content and their adequate transformation in animals are still having many problems.

We made our experiments connected with the above problem in two agricultural big farms, where we produced silage from papilionaceous fodder (alfalfa) rich in proteins by the application of bacterium preparations and organic acids. The stacks weighing more than 100 tons were opened after a five month seasoning. We took samples in every two weeks. Our aim was to establish how the quality of silage changes under the influence of preservatives and as a function of time. Only the most important component indices of silage (dry matter, pH, lactic acid and volatile fatty acid content) were examined. We also made a three-month feeding experiment with dairy cows where the effect of silage on the production, protein and fat content of milk was studied.

The Hungarian "Chinosyl" proved to be the best of the bacterium cultures. Using organic acids (formic acid, propionic acid and their mixture) we have got silage of almost the same quality.

The quantity of the milk produced was not reduced by any one of the fodders, while the fat content increased in most cases. Another reassuring finding was that the quality of silage did not deteriorate by the end of the experiment.

REDUCTION OF NUCLEIC ACID CONTENT IN SACCHAROMYCES CARLSBERGENSIS

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The high content of nucleic acids (NA) (6-10%), mostly ribonucleic acids (RNA) is a major limitation to the greater use of yeast protein in human food. Consumption of yeast protein containing high amounts of NA can cause an increase of uric acid in the blood which may cause gout. According to clinical investigations the daily NA intake should not be higher than 2 grammes. The ratio of crude protein to RNA should be higher than 25; this means that RNA content has to be lower than 2% of dry weight. Chemical and enzymatic methods are known for reducing NA content in yeast.

A simple method has been developed to reduce NA content of brewer's yeast (Saccharomyces carlsbergensis). For the reduction of RNA content the heat-precipitation of yeast protein at alkaline pH seems to be a method suitable for application on a large scale. Addition of NaCl improves the removal of RNA and promotes the precipitation of the proteins at relatively lower temperatures. After mechanical disintegration of the yeast cells, the 5-10 w/v biomass suspension was treated in a three-step heating process. The successive steps involved (1) a heat-shock of a few seconds at 70°C (2) incubation at 40°C for one hour and (3) incubation at 50°C for 2 hours. In yeast cells RNA is localized in the nucleus, mitochondria, ribosomes, and cytoplasm as well. It was found that the optimum concentration of NaCl was 3% (w/v) in the heat-shock process. It seems that the ribosomal RNase was activated in the presence of NaCl. About 80% reduction of NA content was obtained under the conditions mentioned. This process provides a new possibility to increase the amount of brewer's yeast used in food supplementation and complementation.

INVESTIGATION INTO THE RELATIONSHIP BETWEEN FUNCTIONAL PROPERTIES AND DEGREE OF HYDROLYSIS OF FOOD PROTEINS

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Application of a proteinase is often an attractive means of obtaining better functional properties of food proteins without impairing their nutritional value. The degradation of the protein into peptides generally renders the product more soluble, especially at the isoelectric point. However, other functional properties are also affected and, by selecting the proper set of hydrolysis parameters, specific improvements in performance can be achived. The degree of hydrolysis (DH), is defined as the ratio of the number of peptide bonds cleaved (per weight unit) to the total number of peptide bonds (per weight unit). Experiments were carried out with a pH-stat, which presents the most convenient means of controlling DH during hydrolysis reactions carried out at alkaline pH values. The pH was monitored and the hydrolysis mixture was continuously titrated with a base to keep pH constant. Hydrolysates were prepared from milk protein and haemoglobin by treatment with alcalase at pH=8,0, at 50°C.

We have studied a number of functional properties (emulsifying activity, emulsion stability etc.) of proteins of different DH. On the basis of our results we assume, that the DH is an important reaction parameter as it has been shown that the functionality of the resulting hydrolysate is largely determined by DH.

HUNTING FOR MICROBIAL PROTEASES SUITABLE FOR THE PREDIGESTION OF FEEDSTUFFS

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In order to obtain microorganisms with effective and stable proteolytic activity and relatively inexpensive for the predigestion of feedstuffs we isolated and screened 243 Streptomyces strains from different soil samples. Two of the strains isolated so far (KSZ-38 and KSZ-106) possessed high proteolytic activity. We could increase proteolytic enzyme production by shaking the strains selected in submerse culture, on casein medium at 28°C for 96 hrs (n=400/min.). For quick determination of the proteolytic activity we developed a casein-agar diffusion method. After shaking, the mycelia were removed by filtration, then the filtrate was freeze-dryed. Proteolytic activity of the filtrate was determined by azocasein substrate. The proteolytic activity of the redissolved material (distilled water, pH adjusted to 8.0) was 90 per cent of the original activity. This did not change after 24 hrs storage at room temperature. According to the determination of the pH optimum, at least two different proteolytic components were present, with pH optimum at 6.5 and 8.0, respectively. Our previous results suggest that these Streptomyces strains look like to be suitable for practical purposes in proteolytic pretreatment of feedstuffs.

CHANGES IN PEROXIDASE ACTIVITY OF BROCCOLI DURING PROCESSING AND STORAGE IN FROZEN STATE

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Discolouration of the stem parts of deep-frozen broccoli as observed occasionally after prolonged storage at -18°C has been found to be related to elevated peroxidase (POD) activity. Therefore we studied the distribution of soluble, ionically cell-bound and covalently cell-bound activities of the enzyme in the fresh vegetable as well as their changes during processing (blanching and deepfreezing) and subsequent storage at -18°C.

In the fresh state, the total POD activity in the stem was about 1.6-fold higher than that measured in the florets. Soluble POD activity was found to be the main fraction in the stem parts and ionically bound activity in the florets of fresh broccoli. Blanching (95°C, 4 min) reduced total POD activity in stem and florets by one and two orders of magnitude, resp. Freezing brought about a further decrease. Owing to the high initial values, the residual activities were still not negligible. The covalently bound fraction proved the less resistant and the ionically bound fraction the most resistant to processing operations in both parts of the vegetable.

During storage in frozen state the activities decreased, except for the soluble activity in the stem which remained practically constant. The decrease was more marked in the florets. The results permit the conclusion that the soluble enzyme fraction might be involved into the discolouration of the stem parts of deepfrozen broccoli after prolonged storage.

CHANGES IN THE POLYPHENOL-POLYPHENOL OXIDASE COMPLEX AND IN PEROXIDASE ACTIVITY OF PLUM CULTIVARS DURING RIPENING AND STORAGE IN FROZEN STATE

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According to preliminary investigations, enzymatic browning of quickfrozen plums can be related to the catechin content of the fruit. In order to examine this problem polyphenol oxidase (PPO) and peroxidase activities, chlorogenic acid and catechin contents were investigated three times during ripening, then during freezing and frozen storage in samples of two plum cultivars (Besztercei, Olaszkek) important in Hungary.

Soluble and total PPO activities were higher in Besztercei than in Olaszkek. Total PPO activity considerably decreased during ripening in both cultivars between the first and second sampling; soluble PPO activity increased mainly between the second and third sampling. During freezing and storage in frozen state for 2 weeks the activities of both enzyme fractions decreased.

Soluble peroxidase activity in green state did not differ in the two cultivars. During the further period of ripening and freezing as well as frozen storage it was considerably lower in Olaszkek than in Besztercei.

Total polyphenol content was higher during ripening in Olaszkek, the catechin content of Besztercei entirely disappeared when the covering colour was formed.

According to our results the cultivar Besztercei is, owing to its endogenous substrate composition, more suitable for processing in the deepfreezing industry than Olaszkek.

DISTRIBUTION OF MAGNESIUM AND ITS ROLE IN TOMATO PLANTS INFECTED WITH AGROBACTERIUM TUMEFACIENS

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During Agrobacterium tumefaciens infection the Ti-plasmide of bacteria incorporates into the host-plant genome. This process induces a rapid synthesis of 3-indolacetic-acid and trans-zeatine-riboside. The increased level of these compounds leads to increased cell proliferation and finally to formation of crown gall tumor. At the same time, the abscisic acid (ABA) synthesis in infected plants also increases. It is probable that the gibberellin level decreases, or, at least, its effect is supressed by ABA. Growth of plants stops and they remain short. Plant growth and, therefore, the proliferation require increased RNA and protein synthesis and enhanced Mg-levels. We have studied the Mg-content and its distribution in healthy and infected Tomato plants. We found that the Mg-concentrations in "healthy" stalks of infected plants were higher by 20%, in stalks around tumors by 93% and in tumors by 140%, than in healthy plants. Mg-concentration was higher in older tumors than in younger ones.

The role of Mg in plant hormone-, RNA- and protein synthesis will be discussed.

EFFECTS OF MAGNESIUM-DEFICIENCY ON CHLOROPLASTS AND MITOCHONDRIA IN RED PEPPER LEAVES

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The effect of magnesium deficiency on the photosynthetic O_2 evolution and respiratory O_2 uptake was studied. Red pepper (Capsicum annuum var. "Javitott Cecei") seedlings were grown on Mg-free perlit in a greenhouse. The plants were watered with a solution containing either 0.1% magnesium salt or no Mg. The ultrastructure of chloroplasts and mitochondria were studied by electron microscopy. O_2 measurements were carried out on leaf discs with a Clark-type oxygen electrode.

In the early stage magnesium deficiency caused no visible effect in the ultrastructural pattern of chloroplast membranes but the oxygen evolution was low. Ultrastructure of mitochondria and respiration rate were similar to the normal, while the mitochondrial scores in sections were high. At a more advanced stage of magnesium deficiency the chloroplasts were disintegrated in parallel with a further functional breakdown. Starch grains appeared to be intact. There was a decrease in the number of mitochondria and O_2 uptake.

These data show that magnesium deficiency restrains the photosynthetic activity in chloroplasts. Increasing the number of mitochondria in the early stage of magnesium deficiency may be an attempt of the plant to compensate for the energy shortage in the cell.

THE EFFECT OF GAMMA IRRADIATION AND STRESS ON THE RNase AND PEROXIDASE ISOENZYMES OF PEA AND BARLEY

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Gamma irradiation of pea seeds and seedlings increased the activity of degradative enzymes (e.g. RNase, peroxidase, protease) (G. Bagi, E.J. Hidvegi, Biochim. Biophys. Acta 741 55, 1983). In this work we compare the effect of irradiation with various kinds of stress on the isoenzyme patterns of ribonuclease and peroxidase in pea and barley seedlings.

Seeds and seedlings of pea and barley have been irradiated by zero to 300 Gy doses of ^{60}Co γ-radiation and examinations were carried out on the shoots of one week old seedlings. The stress effects used were ageing and mechanical injury. Isoenzymes were separated by polyacrylamide gel electrophoresis and their activity was assayed by staining. It was found that the irradiation specifically altered the isoenzyme pattern. In control pea two main RNase isoenzyme bands were found with Mw of 17,000 and 22,500. After irradiation two more isoenzymes appeared with Mw of 21,000 and 30,000. In control barley 7 peroxidase isoenzyme bands were separated and on irradiation two new isoenzymes appeared, and the activity of two others increased. On the effect of stress the isoenzyme pattern altered similarly. Cytokinins (e.g. kinetin) inhibited the increase of RNase activity caused by irradiation and stress.

ROLE OF BUTYRATE IN THE DEVELOPMENT OF EPITHELIAL KERATINIZATION

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Hyperkeratosis of rumen mucosa, a frequent disorder of intensively fed ruminants, influences negatively the performance of the affected animals. This can be explained by the fact that the ruminal absorption decreases under hyperkeratotic conditions. In a previous publication we suggested the involvement of the high butyrate level of rumen fluid in the induction of abnormal rumen epithelial keratinization accompanied by altered cell-division rate.

The present work was undertaken to study the effect of butyrate on epithelial keratinization. In the first phase, 5 mmole/l sodium n-butyrate was added to primary ruminal epithelial cell culture. An about twofold increase in both cellular protein content and ^{14}C-glycine incorporation was observed. The increased number of rhodanile-positive cells indicated also an advanced keratinization.

Apparently the stimulation of keratinization by butyrate is not limited to rumen epithelium, since homologous organs with similar lining epithelium also show changes when the tissue is exposed to butyrate. Chicks fed butyrate exhibited hyperkeratosis in the crop epithelium and the incorporation of ^{3}H-thymidine into crop epithelial DNA decreased significantly.

On the basis of the foregoing observations, we attempted to prevent the development of ruminal hyperkeratosis in fattening lambs. The basal diet of lambs was suplemented with monensin-Na, 12 mg/kg. Monensin depressed ruminal butyrate concentration and a considerable reduction of the frequency and severity of ruminal hyperkeratosis was observed.

EFFECT OF PATHOGENS ON THE YIELD AND COMPOSITION OF SUNFLOWER SEEDS

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Diseases caused by fungi influence the seed yield, seed components, oil content and quality of oil of sunflower. The aim of our investigations was to obtain information on the changes of important quantitative and qualitative parameters incited by the fungal pathogens which are wide-spread in Hungary. The following parameters were determined: head diameter, thousand achenes weight, kernel-hull ratio, germination ability, oil and protein content, amount of chlorogenic acid, item the qualitative features of oil (composition of fatty acids, acid number, peroxide number, iodine value and tocopherol content).

For the investigations seed samples of two new Hungarian hybrids (Koflor-2 and IBH-166) were gathered in the experimental field of Bacsalmas State Farm on the 31st of August 1983 in the stage of yellow ripening (IBH-166) and perfect maturity (Koflor-2). Diseased plants were selected on the basis of visible disease symptoms: 1. Stem diseases caused by Alternaria helianthi, A. zinniae, Phomopsis helianthi and Sclerotinia sclerotiorum, 2. Head decay caused by S. sclerotiorum and Ph. helianthi (the latter one in the case of IBH-166 hybrid only). Samples were collected from healthy plants as controls.

From the data obtained it could be concluded that oil and protein accumulation in the seeds was reduced mainly by the fungi which cause stem diseases leading to inhibition of nutriment-transport, while the germination of seeds was not injured. Viability of kernels was decreased by the pathogens which cause head rot, but the amounts of reserve protein and oil were not modified significantly. The amount of chlorogenic acid usually increased up to 110-120% in consequence of infections.

INFLUENCE OF HERBICIDES ON THE GLUTATHIONE-S-TRANSFERASE LEVELS OF MAIZE SEEDLINGS

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The selective toxicity of a herbicide to different plant species may be influenced by numerous factors, of which the ability of the plant to detoxify the herbicide is possibly the most important. Thiocarbamate and chloroacetamide herbicides applied to maize (Zea mays L.) are detoxified by the crop plant via conjugation with glutathione. This reaction may be non-enzymatic or mediated by the enzyme glutathione-S--transferase (GST) (F. Dutka, T. Komives, IUPAC Pesticide Chemistry (Ed. J. Miyamoto) Vol. 3, p. 213, Pergamon Press, 1983; T. Mozer, D.C. Tiemeier, E.G. Jaworski, Biochemistry 22 1068, 1983).

In exploring the above detoxification system against herbicides we studied GST levels in maize (hybride 3950, Szeged) treated with EPTC (S--ethyl-N,N-dipropyl thiocarbamate) and acetochlor (2-chloro-N-(2-ethyl-6--methylphenyl)-N-ethoxymethyl acetamide). Herbicide treatments were carried out in sand culture (F. Dutka, T. Komives, IUPAC Pesticide Chemistry (Ed. J. Miyamoto) Vol. 3, p. 213, Pergamon Press, 1983). GST enzyme activities were measured spectrophotometrically using 1-chloro-2,4-dinitrobenzene as substrate (T. Mozer, D.C. Tiemeier, E.G. Jaworski, Biochemistry 22 1068, 1983).

Time-course and dose-response data indicate that the treatments with the above herbicides result in significantly elevated levels of GST in maize seedlings, thereby increasing their capacity to detoxify these herbicides.

THIOCARBAMATE HERBICIDE EFFECT ON FATTY ACID COMPOSITION OF MEMBRANE LIPIDS OF CORN PLANTS

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The metabolic action of the thiocarbamate herbicide EPTC (S-ethyl--N,N-dipropyl thiocarbamate) in higher plants has been extensively studied. Though this herbicide is known to affect epicuticular wax and fatty acid biosynthesis in several plants, there are indications that EPTC has no such effect on corn (Zea mays L.) which is the main crop for EPTC use.

To clarify the possible role of EPTC in membrane lipid biosynthesis of corn, we have analyzed the incorporation of (1-^{14}C)acetate into lipid classes (neutral lipids, galactolipids and phospholipids), and the fatty acid composition of leaf and root lipids. Extraction and analysis of lipids from 14-day-old corn plants (hybrid JX-SC 92, Martonvasar, Hungary) were performed by standard methods (I.Cs. Barta, T. Komives, F. Dutka, Radiochem. Radioanal. Letters 58 357-362, 1983; I.Cs. Barta, T. Komives, J. Chromatogr. In press).

Phytotoxic doses of EPTC do not induce changes in the total labeling of the membrane lipid fraction of leaves and roots, whereas the ratios of label incorporation from (1-^{14}C)acetate into various lipid classes are significantly altered by the herbicide. Fatty acid composition data show that EPTC treatment leads to the inhibition of corn leaf linolenic acid biosynthesis and elevation of root palmitoleic acid content.

These results suggest that EPTC has a direct effect on the membrane lipid biosynthesis of corn plants, resulting in symptoms of phytotoxicity.

PLANT PHYSIOLOGY OF ACETOCHLOR

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The α-chloroacethamide herbicide acetochlor (2-chloro-N-(2-ethyl-6--methylphenyl)-N-ethoxymethyl acetamide) is widely used in the Hungarian agriculture for controlling annual grasses and certain broadleaf weeds in corn (Zea mays L.).

On the basis of available results (E.G. Jaworski, J. Agr. Food Chem. 17 165, 1969) the selectivity of this herbicide can primarily be attributed to the differential rates of its metabolism (detoxication) in sensitive and tolerant plant species. For this reason we made comparative studies on the plant (Zea mays L., Triticum aestivum L.) physiology of this herbicide by using carbonyl ^{14}C labeled acetochlor (I. Jablonkai, A.F. Marton, F. Dutka, Radiochem. Radioanal. Letters 53 253, 1982).

We have found that i/ the amount of acetochlor absorbed increased with increasing herbicide tolerance of plants; ii/ acetochlor translocation was retarded in tolerant corn plants as compared to the sensitive wheat plant; iii/ in accordance with the herbicide susceptibility of the plants the majority of acetochlor absorbed was transformed to water-soluble metabolites in a short time (6 hr); iv/ glutathione conjugation was the definitive pathway of metabolism.

INHIBITION OF PHOTOSYNTHESIS BY 4-HYDROXY-BENZONITRILE DERIVATIVES OF HERBICIDAL CHARACTER

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Photosynthesis inhibiting properties of some 4-hydroxy-benzonitrile derivatives, developed and synthesized by Chinoin Pharmaceutical and Chemical Works Ltd. (patent N° 1340 and 1341/82.), were investigated in in vivo and in vitro photosynthesizing systems, to clarify their mode of action. The following compounds were investigated: bromoxynil, 3-nitro-4--hydroxy-5-bromo-benzonitrile, methylcarbonic and i-propylcarbonic esthers of this latter compound. In vivo spinach leaves and 7-day-old wheat leaves, in vitro chloroplasts (type C) isolated from spinach or wheat leaves were used for determination of the inhibition of photosynthetic electron transport.

It was proved that the two esther derivatives - like bromoxynil and 3-nitro-4-hydroxy-5-bromo-benzonitrile - inhibit the photosynthetic electron transport on the reducing side of photosystem II between Q and the DCPIP site. pI_{50} value - characterizing the in vitro inhibition of photosynthetic activity - of spinach chloroplasts for methylcarbonic esther derivative was: 4,36, for i-propylcarbonic esther was 4,16. The CO_2 fixation of spinach leaves in vivo was inhibited by all compounds in a concentration of 30 μmole l^{-1} after 2 h treatment. The inhibition was 89% in case of bromoxynil and methylcarbonic esther of 3-nitro-4-hydroxy--5-bromo-benzonitrile, and 75% in case of i-propylcarbonic esther.

CO_2 fixation of wheat leaves was inhibited significantly (52%) only by bromoxynil. There is a difference between the sensitivity of the two model plants even on the level of chloroplasts.

SOIL ADSORPTION AND DECOMPOSITION OF SOME NEW PESTICIDES

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The adsorption and mobility of the first metabolite of fenitropan (1-phenyl-2-nitro-3-acetoxi-prop-1-ene, Comp.I.) and ciobutid (2-cyano-2--phenyl-butanamide, Comp.II.) have been determined on two Hungarian soils (provenance: Erd and Martonvasar). Fenitropan (2-nitro-1-phenyl--trimethylene-diacetate, Comp.III.) and Comp.I. were exposed to UV light and the rate of decomposition was measured.

Higher quantities were adsorbed from Comp.II. than from Comp.I. on both soils. Good linear correlations have been found between the adsorbed quantity (μg per g soil) and the equilibrium concentration (ppm) proving that in the concentration range applied (up to 250 μg per g soil) the pesticides occupy only a small part of the adsorptive capacity of soils. Comp.I. showed negligible mobility and strong decomposition on soils even during the relatively short period of time of the experiment (8 hours). Comp.II. moved better, especially on the Martonvasar soil. Both compounds were found to be sensitive to UV light. Their decomposition followed first order kinetics; about 50% of both compounds decomposed in the first sixty minutes. No significant differences were found between the decomposition parameters of the compounds.

Comp.II. plant growth regulator (PHORBAN^{R}-80WP, 80% ciobutid)
Comp.III. contact fungicide (VOLPAROX^{R}-20EC, 20% fenitropan)

APPLICATION OF BIOCHEMICAL ENERGY-VALUES FOR APPROACH OF VEGETAL PRODUCTIVITY

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A biochemical approach was worked out to estimate the plant productivity and its relations to mass, chemical constituents and energy value on the evidence of biosynthetic courses (pathways) of vegetal organic matters, in equivalents to simple carbohydrate (in mass and in energy) (ME: minimal equivalent; 1/ME: production equivalent) (Nehez: Acta Biochim. Biophys. 17 1-2, 139, 1982; Nehez: Acta Aliment. 13 2, 117-134, 1984).

Energy-values are calculated from enthalpies and electron equivalents for vegetal carbohydrates, fatty acids, sterols and amino acids. For the reduction of nitrate and sulphate 960.5 and 960.5 kJ (2/6 glucose) per N and S were used. The enthalpy +input(reduction) for amino acids are (in kJ/g) for gly 20.666, ala 24.430, ser 19.347, cys 24.137, cyn 23.212, asp 16.375, asn 23.346, thr 22.098, pro 28.738, glu 19.184, gln 25.502, val 29.890, met 27.181, his 31.388, leu 31.728, ile 31.717, lys 33.126, arg 34.738, phe 31.648, tyr 27.721 and trp 33.235. The mass equivalents (1/ME: g matter/g simple carbohydrate) and energy-values (kJ/g matter) are for starch 0.90 and 17.362, in general for vegetal carbohydrate 0.87 and 17.675, fibre 0.80 and 18.614, oil 0.34 and 39.575 and protein (from nitrate and sulphate) 0.40 and 30.685 (enthalpy +input/reduction). These values are demonstrated for detailed chemical composition of plant seeds.

The relation of mass equivalents and energy-values seems to be contradictory, but biochemically they are selfevident. From the biochemical energy-values arises a practical conclusion, that vegetal productiveness can be increased by the mass yield, its chemical composition and energy--values, equally.

POSSIBILITY OF SPONTANEOUS FORMYLATION REACTION BY ENDOGENOUS FORMALDEHYDE AFTER HEAT SHOCK IN PLANTS

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Earlier we have found that one of the early events of plant response to heat shock is the accumulation of formaldehyde that is produced in direct proportion to increasing temperature.

It is obvious that this simple aliphatic aldehyde can take part in various reactions in plant tissues. According to our investigations the formaldehyde is capable to formylate spontaneously the primary amino groups (e.g. ε-NH_2 group of L-Lys) without any catalyst (E. Tyihak, L. Trezl, I. Rusznak, Pharmazie 35 18-20, 1980; L. Trezl, I. Rusznak, E. Tyihak, T. Szarvas, B. Szende, Biochem. J. 214 289-292, 1983). The essence of this spontaneous chemical reaction is as follows: as a result of the reaction between the formaldehyde and the amino group an addition complex is formed. This very labile, unseparable compound can be stabilized by resonance to an iminium cation (not a Schiff base!). In the next step N-methyl group is formed through the reduction of the iminium cation with a further formaldehyde molecule. In this reaction step formyl cation is produced from the formaldehyde. The formyl cation can react with an amino group of another molecule by which an N-formylated derivative is produced.

It seems that the spontaneous formylation reaction of formaldehyde e.g. with ε-NH_2 groups of polypeptides or proteins may have importance mainly in the protection mechanisms of plant tissues against infections.

EFFECT OF THE ARTIFICIAL PROPAGATION FOLLOWING THERMAL STRESS ON THE ACTIVITY OF SERUM TRANSAMINASES (GOT, GPT) OF CARP (CYPRINUS CARPIO L.)

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Fish can counteract thermal stress by resistance, acclimation with metabolic adjusment, and thermoregulation chiefly by behaviour. There is a need for more complete studies on the effects of temperature on various functions connected with metabolism.

The aim of this investigation was to study how the artificial propagation, following thermal stress affects the activity of serum transaminases of common carp. Thermal stress was modelled by the elevation of temperature from $5^{\circ}C$ to $25^{\circ}C$. The change was gradual in the first group ($5^{\circ}C$ per 12 hours) and rapid in the second one ($10^{\circ}C$ per 1 hour). Before spawning the first group was kept at "maturing" temperature for 1 hour, while the second one was kept for 24 hours. GOT and GPT activities were measured by using standard assay kits (Boehringer).

The activity of GOT and GPT increased significantly in both groups following the thermal stress. The GOT increased from 14,22 $\pm$ 2,34 U/l to 30,01 $\pm$ 3,00 U/l, the GPT increased from 2,70 $\pm$ 0,72 U/l to 4,00 $\pm$ 0,35 U/l in the first group, while in the second group the same parameters changed from 10,07 $\pm$ 1,12 U/l to 61,52 $\pm$ 0,54 U/l and from 3,39 $\pm$ 1,12 U/l to 11,30 $\pm$ 1,31 U/l, respectively. During "maturing" and after hypophysation a decrease of transaminases was observed. A significant increase of the activities followed the spawning of the fish.

Our results show that the thermal stress and the too short "maturing" period caused deep metabolic changes. The increasing of activity of transaminases after spawning could reflect the resorbtion processes.

FATE OF ACETOIN IN HIGHER ANIMALS

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Liver is the organ in mammals and birds where the metabolism of acetoin takes place. The ratio of the amounts of $^{14}CO_2$ resulting from (2-^{14}C) and (1-^{14}C)-acetoin is the same as that from (1-^{14}C) and (2-^{14}C)--acetate, showing a non citric acid cycle origin. The ratio of $^{14}CO_2$ originating from the differently labelled acetoin molecules agrees well with the stoichiometry of the cholesterol biosynthesis.

We could directly demonstrate that the carbon atoms of acetoin incorporate into cholesterol and fatty acids. The extent of incorporation can be increased by the addition of thiamin diphosphate.

The following building block molecules were isolated and identified: acetate, glutamate, proline and ornithine. It is interesting that no detectable amounts of citric acid cycle intermediates could be isolated.

The mechanism by which the C_4-ketol is degraded into C_2-units is not known as yet.

ACYLCARNITINES OF HUMAN URINE: EVIDENCE FOR UNIDENTIFIED CARNITINE ESTERS

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Recent studies have indicated a regulatory function for carnitine in maintaining the acyl-CoA/free CoA ratios in tissues and whole body. Since the acylcarnitine to free carnitine ratio should reflect the acyl-CoA to free CoA level in tissues, changes in the relative amounts of specific acylcarnitines to free carnitine should reflect changes in the respective acyl-CoA pool.

Recently we described a sensitive radiochemical assay for radiolabeling, separating and quantitating individual short-chain acylcarnitines. This method has the potential of being a diagnostic tool for diseases in which major changes in specific short-chain acylcarnitines might occur, such as propionic acidaemia. This methodology has been applied for the investigation of human urine and other tissues. In tissues such as heart, muscle and liver acylcarnitine profiles and amounts were similar to those reported previously using the gaschromatographic technique. However, when the more sensitive methodology was applied for the analysis of human urine, variable amounts, 0-80% of the acylcarnitine content of urine consisted of compounds different from acetyl-, propionyl-, C4- or C5-acylcarnitines. These unidentified acylcarnitines were not detected in urine from individuals who were on total parenteral nutrition, indicating that the unusual acyl portions may be dietary or intestinally derived. The increase in the relative amount of branched-chain acylcarnitines in urine as compared to blood, indicates, that the kidney exerts selective reabsorption of free and acetylcarnitine while the reabsorbtion of the branched-chain acylcarnitines is less effective. Studies on the identification of the unknown urinary acylcarnitines are in progress. These acyl-compounds show greater retention times on gaschromatography and greater lipophylicity on HPLC-columns.

UPTAKE OF CARNITINE BY PERFUSED RAT LIVER

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The role of carnitine (L-3-hydroxy-4-N-trimethylaminobutyrate) in carrying activated fatty acids across the inner mitochondrial membrane to the site of beta-oxidation has been well established. Recently, a regulatory function of carnitine has been proposed since besides other factors an increase in hepatic carnitine concentration is necessary for accelerated ketone body synthesis observed in starvation. In the regulation of hepatic carnitine concentration the transport between the liver and blood stream is probably the most effective factor.

In this study we examined the uptake of carnitine by perfused liver of fed and starved rats. Perfused liver seems to be the most proper model for the sutdy of transport across the liver cell membrane.

The initial observation was that livers of fasted rats took up much more carnitine than those of fed rats. At near-physiological, 62 μM, carnitine concentration the initial velocity of net uptake was 0.018 and 0.038 μmoles $\cdot$ min^{-1} $\cdot$ 100 g body weight^{-1} in "fed" and "fasted" livers, respectively.

The greater ability of "fasted" livers to take up carnitine is due to both a smaller K_m and an increased V_{max} value. The apparent K_m for net carnitine uptake was found to be 1.11 mM in "fasted" livers and 2.52 mM in "fed" livers. The respective V_{max} values were 1.04 and 0.84 μmoles $\cdot$ min^{-1}. 100 g body weight^{-1}, respectively.

The carnitine uptake showed the properties of concentrative transport. The tissue to perfusate concentration ratios were 5.7 and 18.2 in "fed" and "fasted" livers, respectively. The uptake could be inhibited by 59.6% when N_2 atmosphere and 2,4-dinitrophenol were applied together.

PREPARATION OF MALTOOLIGOMERS BY CONTROLLED ENZYMATIC HYDROLYSIS

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The derivatives of maltooligomers are very important and valuable components of diagnostics, moreover there is a possibility to use them as the carbohydrate components of artifical antigens.

γ-cyclodextrin is a very good substrate of Aspergillus oryzae α-amylase, and all of the oligomers from maltose to maltooctaose are detectable in the reaction mixture during the enzymatic hydrolysis. Based on this observation we developed a method for the preparation of each maltooligomer in pure form applying relatively simple separation methods.

The qualitative and quantitative distribution of the maltooligomers was determined by HPLC during the enzymatic hydrolysis. This method allowed us to investigate the kinetics of the enzymatic hydrolysis and to determine those periods of the entire reaction during which certain components were present in relatively high quantity in the mixture.

In this way there is a possibility to prepare the required maltooligomer with the best yield.

AMINO ACID ANALYSIS IN BIOLOGICAL SAMPLES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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Amino acid analysis is frequently used in biochemical investigations. The early methods are based on ion-exchange chromatography of amino acids and a postcolumn ninhydrin derivatization to enhance the sensitivity of detection. This method requires much time, high amounts of solvent and sample. Introducing the High-performance Liquid Chromatography (HPLC), and altering pre- and postcolumn derivatization agents, the analysis time decreased to 30-40 minutes and the detection limit to picomol or femtomol quantity of amino acids.

This work presents a routine RP-HPLC method to allow the analysis of 18 biogenic amino acids within 25 minutes at picomolar level. The method is based on the precolumn derivatization of amino acids with o-phtalaldehyde (in the presence of 2-mercaptoethanol), the separation of derivatives on a reversed phase packing material, and UV and/or fluorescent detection. The gradient eluting systems used contain a non--ionic surfactant (Triton X-100) to improve the sensitivity and the resolution by the micellar eluent. A further advantage of this method is that the preparation of the sample is very simple, because vacuum-dried protein-, or peptide hydrolysates derivatized by OPA can be introduced directly into HPLC, moreover the analysis of free amino acids in cultured cells or in their incubation medium can be performed after deproteinization.

This rapid, routine HPLC method is a promising tool in biochemical studies, since it allows the simultaneous qualitative and quantitative analysis of amino acids in many in vivo and in vitro systems.

A NEW KINETIC UV METHOD FOR THE DETERMINATION OF FACTOR XIII OF BLOOD COAGULATION IN PLASMA

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A new kinetic assay was designed for the estimation of Factor XIII in plasma. Plasma fibrinogen was removed by bentonite treatment prior to the measurements. During the lag phase FXIII was transformed by thrombin and Ca^{2+} into active transglutaminase which catalyzed the binding of the amine substrate ethylamine to a glutamine residue of acetylated, dephosphorylated β casein. During the reaction ammonia was released and continuously monitored in an indicator reaction catalyzed by the NADPH dependent glutamate dehydrogenase. The optimal substrate and activator concentrations were determined. For the elimination of clottable fibrinogen from the plasma samples bentonite treatment was found more advantageous than traditional heat treatment. The method correlated well with the most widely used amine incorporation and immunoinhibition FXIII assays. A reference range of 9.2-17.3 U/L was established. The optimal condition variance of the method was less than 3% in the normal range. The method has several theoretical and practical advantages over traditional Factor XIII determinations.

Fe^{3+}-EDTA COMPLEX ACCELERATES THE ENZYMATIC LIPID PEROXIDATION OF BRAIN MICROSOMES

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LIPID PEROXIDATION IN RAT LIVER SUBMITOCHONDRIAL FRACTIONS

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Lipid peroxidation in rat liver mitochondria can be induced both by Fe^{2+} ions and by a NADPH-dependent system which requires ADP chelated Fe^{3+} ion. The latter process was shown to be inhibited by succinate, and a protective mechanism (dependent on mitochondrial substrate oxidation) against the lipid peroxidation has been assumed (Meszaros et al, Biochim. Biophys. Acta 713 675-677, 1982); although the participation of microsomal contamination in mitochondrial lipid peroxidation has been questioned.

According to our observations various parameters of the mitochondrial lipid peroxidation (time course, requirement of a NADPH-regenerating system, etc.) differ from those of the NADPH-dependent process occurring in microsomes. Mitoplasts and mitochondrial inner membrane fractions also display lipid peroxidation. The NADPH-dependent lipid peroxidation activity of mitoplasts is at least 50% higher than that of intact mitochondria, although the degree of microsomal contamination calculated from glucose-6 phophatase activity is negligible. This process can be induced not only by the ADP-Fe^{3+} but also by EDTA-Fe, containing either Fe^{2+} or Fe^{3+} ions. However, lipid peroxidation induced by Fe^{2+} ions was almost completely inhibited by the chelating agents ADP and EDTA. Succinate reduced this peroxidative activity by 50%.

These observations can be interpreted by supposing the existence of two iron-dependent lipid peroxidation mechanisms operating in mitochondria.

ALTERATIONS OF MITOCHONDRIAL PROTEINS DURING NADPH DEPENDENT LIPID PEROXIDATION

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The peroxidative destruction of membrane lipids seems to be a very important factor in the turnover of membranes and in the cellular ageing. Recently lipid peroxidation processes were described in mitochondria as well. The NADPH-dependent lipid peroxidation (LPO) can be inhibited by succinate.

We have studied the changes in different mitochondrial fractions and functions in response to NADPH-dependent LPO in the presence or absence of succinate. Lipid peroxidation was followed by the estimation of malondialdehyde formed.

The protein patterns of intact mitochondria and submitochondrial fractions were examined with SDS-polyacrylamide gel electrophoresis. Marked differences were detected between control and lipid peroxidated (in the absence and in the presence of succinate) preparations. Some protein bands disappeared after LPO. The latter phenomenon can be attributed to the well known capability of malondialdehyde to cross-link proteins. There was no increase in the lower molecular weight protein region of protein bands. This fact showed that no considerable protein break-down occurred during LPO. The changes in the protein pattern could be partially prevented by succinate.

The changes in the activities of different mitochondrial enzymes e.g. ATP-ase, succinate-dehydrogenease, glutamate-dehydrogenase were monitored during LPO. The succinate utilization decreased to 40% of the control value. On the other hand the cytochrome oxydase activity remained unaffected. The protective effect of succinate on the activity of enzymes was demonstrated, too.

ISOLATION AND PARTIAL CHARACTERIZATION OF MITOCHONDRIAL AND CYTOPLASMIC MYOSINS FROM RAT AND HUMAN LIVER

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In the previous studies mitochondrial myosin fractions were prepared from bovine parotis (MET 48. Vandorgyules, Debrecen, p. 64, 1983) and compared with rabbit muscle myosin (m. long. dorsi).

The mitochondria have their own DNA and capacity of protein synthesis (2-3 endogen ribosomes). They are able to carry out micropulsations (four types of movements). Therefore, several mitochondriologists suggested that the phyloorganogenesis of mitochondrium took place in an endogenic, according to others in a symbiotic manner.

The existence of contractile proteins was also suggested, and the purification of actomyosin-like proteins was attempted twenty years ago already. Naturally, the early preparations were far from the present purified preparations performed by up-to-date techniques. In addition, myosins prepared at the present time contain various types of isoenzymes even when they originate from a certain tissue, from a single muscle type or a single fibre.

We tried to prepare cell-myosin from mitochondria and nucleus-free cytoplasm of human and rat liver. It was found that they differed in their phospholipid content, ATPase activity, capacity of Cu^{2+} and actin-binding. We think that myosin preparations from liver cells are also heterogeneous; they can originate not only from the contractile elements of the cytoskeleton, but from those of the mitochondrial structure. So, the phyloorganogenesis of mitochondria could be explained by the symbiotic theory.

THE SEPARATION OF DIFFERENT TYPES OF BOVINE RETINAL MITOCHONDRIA AND THEIR MEMBRANE FLUIDITY

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Bovine retinal mitochondria can be divided into two groups according their size and function. Mitochondria of the photoreceptor inner segments represent the larger type and they take part in the continuous renewal of the photoreceptor outer segments discs. Mitochondria located in the other retinal neurons are smaller and these compartments play an essential role in the neuronal, general metabolism.

We tried to separate subcellular fractions rich in either of the two types of mitochondria. After repeated centrifugations the fraction of 1.5 g/cm^3 sucrose density was rich in smaller mitochondria (0.3-0.5 μm average diameter) and the pellet below 1.22 g/cm^3 sucrose density contained mainly larger mitochondria (0.6-1.1 μm) when run at 75,000 g for 16 h.

Mitochondrial fractions separated from dark or light adapted retinas were labeled by 5 doxyl stearic acid to determine the temperature dependency of the order parameter (S). Though the changes in this parameter were very similar in both types of dark adapted mitochondria the S values were greater in the larger mitochondrial fraction indicating that these membranes are more densely packed. Light adaptation resulted in a more pronounced ordering of the latter fraction (first of all above 30°C) than that of the other neural mitochondrial fractions.

MITOCHONDRIAL ALTERNATIVE RESPIRATION IN THE CELL CYCLE

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The mitochondrial cyanide-resistant, salicylhydroxamic acid (SHAM) sensitive respiration of unknown biological function was studied in synchronous tissue (potato) and cell (C. tropicalis) cultures. The changes in the actual activity (inhibited by SHAM in the absence of cyanide) and in the total capacity (measured in the presence of cyanide, sensitive to SHAM) of the alternative respiratory pathway were followed during the cell cycle by polarographic measurement of oxygen consumption.

We observed that the cyanide-resistant respiratory capacity is induced in the second cell layer of a slice cut from a potato tuber where cell division occurs under proper conditions. The actual alternative respiration can be observed in proliferating tissue slices and in the G_1 phase only. We suppose that auxins are responsible for activating the alternative pathway.

Results of our experiments on yeast cells were evaluated using the Transition Probability Model. We have found that alternative and cyanide-resistant respiration are characteristics of cells being in the stochastic, so called "A" phase of cell cycle (which is a part of G_1). Thus the high cyanide-resistance of a resting cell population can be explained by the high ratio of "A" phase cells. The cyanide sensitivity of the fast growing asynchronous cells is the result of few "A" phase cells present in these populations.

EFFECTS OF EQUISETIN ON MITOCHONDRIA

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The effects of equisetin[1,2] - an N-methyl tetramic acid chromophore containing antibiotic, produced by Fusarium Equiseti (Corda) Saccardo - on rat liver mitochondria have been studied.

The following results have been obtained: 1. In has no effect on the basal ATP-ase activity of mitochondria (measured in the absence of 2,4-dinitrophenol = DNP). 2. It inhibits the DNP-activated ATP-ase activity in a concentration dependent manner (50% inhibition at about 6 nmoles equisetin/mg mitochondrial protein). Mg^{2+}-ions do not influence this inhibition. 3. It inhibits mitochondrial oxygen uptake activated either by ADP or DNP in a concentration dependent manner, using glutamate+ +malate as a substrate pair. 4. It does not significantly influence the K^{+}-Na^{+}-ATP-ase activity of a rat brain synaptosomal preparation even at two orders of magnitude higher concentration than that needed to inhibit DNP-activated mitochondrial ATP-ase activity by 50%.

From the above mentioned results we suppose that equisetin is an inhibitor of mitochondrial proton-pumps. Experiments are under way to test the validity of this supposition.

1/ H.R. Burmeister et al, Antimicr. Agents and Chemoth. 5 634, 1974

2/ R.F. Vesonder et al, J. Antibiotics 32 759, 1979

CHANGES IN THE OXYGEN BINDING OF ERYTHROCYTES DUE TO CERTAIN EXTRACELLULAR STIMULI

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The basic function of erythrocytes, the oxygen binding, quickly responses to certain changes in the organism, generally attributed to their influence on the metabolism of the cell. Earlier observations pointed out that changes in the oxygen affinity of erythrocytes could be induced by ligands of some receptors on the external surface of the red cell membrane, i.e. a considerable increase in erythrocyte oxygen affinity was found due to the effect of adrenaline. In the present work, the effect of acetylcholinchloride (AcChCl) and its analogues on the oxygen transport capacity of erythrocytes was demonstrated, in the concentration range of 10^{-5} - 10^{-8} M, where an increase of 10-30 per cent was revealed in the oxygen affinity values. The effect of AcChCl could be observed definitely only when its splitting enzyme acetylcholinesterase had been inhibited, while its analogue, the carbamoylcholinchloride, showing very slow hydrolysis, induced directly a significant effect. Since the ligands used in these experiments did not cause significant changes in the 2,3 DPG level or in glycolytic activity, their effect could not be explained simply by an influence on the erythrocyte metabolism.

STIMULATION DEPENDENT ARGINASE SECRETION OF PERITONEAL MACROPHAGES

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Intracellular arginase is a key enzyme in amino acid metabolism. Macrophages are able to secrete this enzyme into their environment. Resident and stimulated rat and murine peritoneal macrophages equally produce arginase.

The arginase secretion of macrophages stimulated in vivo by casein, exceeds the enzyme production of resident and thioglycollate-elicited cells as estimated by ornithine release.

The enzyme is secreted by murine peritoneal macrophages both in Hanks and Eagle medium during the 24 h cultivation period. The intracellular arginase activity of the macrophages exceeds that of the secreted amount, especially in adherent cells.

Rat peritoneal exudate cells reutilize the released ornithine. Therefore arginase could be detected only after the removal of the macrophages and adding exogenous arginine. This phenomenon was not observed in murine cells. The arginase production is enhanced by the addition of exogenous arginine during the 24 h cultivation period.

The biochemical characteristics of arginase produced by macrophages were compared to those of arginase purified from liver.

The arginase secretion might have a distinct role in cell cooperation by regulating the polyamine synthesis and/or amino acid metabolism.

EFFECT OF FRUCTOSE ON ANABOLIC PROCESSES OF ISOLATED HEPATOCYTES

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The effect of fructose (10-40 mM) on the incorporation of ^{14}C-valine and ^{3}H-uridine into trichloroacetic acid insoluble material of isolated hepatocytes and on gluconeogenesis was investigated. Isolated hepatocytes were prepared from mouse liver with the collagenase perfusion method. The cells were incubated in a Krebs-Henseleit bicarbonate buffer, containing pyruvate (5 mM), albumin (1%), amino acids necessary for protein synthesis (1 mM each) and glucose (8.5 mM) under constant bubbling of gas (O_2:CO_2, 95:5 v/v) at 37°C. In the experiments where gluconeogenesis was studied glucose was omitted from the incubation medium.

In the presence of fructose the amino acid incorporation was reduced to 80-85% of the control value in a dose dependent manner after 60-180 min incubation. 40 mM fructose caused an even stronger inhibition temporarily. This inhibition became manifest about 30 min after the beginning of the incubation and lasted for 5-10 minutes. When glucose was omitted from the medium the inhibition of amino acid incorporation was not observed in the presence of fructose.

^{3}H-uridine incorporation of isolated hepatocytes was decreased by 10-40 mM fructose to 85-90% of the control value. These data are in accordance with earlier results of several laboratories according to which the addition of fructose in high concentrations was accompanied with a temporary ATP deficiency.

The effect of fructose on the hepatotoxic action of different drugs metabolized in the liver was also examined and will be discussed.

BINDING OF α_2-MACROGLOBULIN TO ISOLATED HEPATOCYTES

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In our earlier experiments a specific binding of thrombin--antithrombin-III complex (T-AT) to isolated hepatocytes has been observed. α_2-Macroglobulin (α2M) is also assumed to have a role in the inactivation of various haemostatic proteases, therefore we examined the binding of thrombin-α2M and plasmin-α2M complexes to isolated hepatocytes. ^{125}I--labeled proteins were used for this purpose. Binding of thrombin-α2M or plasmin-α2M to the cells was concentration and time dependent and it was 3-4 times higher than the binding of α2M alone. Addition of a 50-fold excess of the unlabeled α2M-protease (thrombin or plasmin) complex resulted in a 80% decrease of the binding of the labeled complex. In the course of a 180 min. incubation internalization of the complexes was 35-40%, while in the case of α2M added alone, it was 15-20%. The inactivation of α2M by hydroxylamine did not alter its binding to isolated hepatocytes. There was no change in the binding of the inhibitor-protease complex when the single constituents were added in a great excess together with the labeled complex. Our results suggest that both components of the complex (thrombin and α2M or plasmin and α2M) contribute to the formation of the binding sites of the inhibitor-protease complexes. In a series of further investigations no competition was found between the binding of T-AT and thrombin-α2M complexes. Therefore we assume that the binding of these complexes should take place at different sites of hepatocytes.

INTERACTION OF GLU- AND LYS-PLASMINOGEN, PLASMIN AND ANTITHROMBIN III WITH ENDOTHELIAL CELLS

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Interaction of ^{125}I-labeled human plasminogens (Pg), plasmin (P) and antithrombin III (ATIII) with cultured bovine and mini pig aortic endothelial cells were investigated. Differences were found between the binding parameters of the two forms (Glu- and Lys-) of Pg, and between Pg and P. The cells were saturated with P within 2 min, whereas Pg binding occurred much slower; the amount of bound P was 8 times higher than that of Pg as measured with mini pig endothelial cells. The rate of release was similar for P and Pg. The amount of the bound Lys-Pg increased during a 120 min. incubation period, whereas that of the bound Glu-Pg did not change considerably. 1 to 50 nM 6-aminohexanoic acid decreased the binding of both P and Pg and increased the dissociation of bound proteins suggesting that the Lys-binding site(s) of P was involved in the interaction between the enzyme and the cell. α_2-Macroglobulin, in a molar excess to enzyme, also inhibited the binding of P to endothelial cells, and increased the release of bound P by forming an enzyme-inhibitor complex which did not bind to these cells. In addition, ATIII also bound to endothelial cells. At a concentration of 0.5-1 μM ATIII, a near physiological inhibitor level, maximal binding occurred within 10 min.

EFFECT OF IMMUNOSUPPRESSORS ON THE IMMUNOGEN INDUCED ENDONUCLEASE

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The Ca^{2+}, Mg^{2+} dependent endonuclease of nuclei is tightly associated to the chromatine, and its splitting activity is limited to adjacent regions of DNA. The enzyme cleaves the DNA in the linker regions between nucleosomes.

The induction of Ca^{2+}, Mg^{2+} dependent endonuclease in nuclei isolated from lymphoid tissues (spleen, thymus) by immunization has been shown by us to be dependent on the dose of the antigen, and on the time elapsed after the immunization (F. Antoni, I. Csuka, N. Khodarev, I. Votrin. FEBS Abstract, Moscow 1984).

The minimal dose of sheep red blood cells necessary to elicit an increase of Ca^{2+}, Mg^{2+} dependent endonuclease activity was 10^6 cells. The enzyme induction as well as the production of specific 19S-IgM type antibodies was abolished by the immunosuppressive substances mercaptopurine and thioguanine, and also by emetine which has previously been found to inhibit the immune response (I. Csuka, F. Antoni, Biochemical Pharmacology, accepted for publication, 1984). The biochemical changes related to the elevation and suppression of the endonuclease activity in the various stages of the immune response were investigated.

The Ca^{2+}, Mg^{2+} dependent endonuclease of nuclei plays a role in the proliferation and differentiation.

A relationship between the increasing activity of Ca^{2+}, Mg^{2+} dependent endonuclease, the changes of the immunochromatine and the synthesis of specific antibodies is suggested.

IMMUNOMODULATING FACTOR FROM PERITONEAL EXUDATE CELLS

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A heat-stable factor inhibiting ^{3}H-thymidine incorporation of lymphocytes was isolated from the serum-free culture medium of mouse peritoneal exudate cells induced by ip. administered casein (I. Csuka, F. Antoni, A. Takats, FEBS Abstracts, Moscow 1984).

The factor appeared in the 2nd hour of culturing and the maximum activity was found at 24 h. The production of the factor was not influenced by emetine and other inhibitors of protein synthesis, which suggested that the factor was present in the cells in a preformed state, and it was activated during incubation. Both spontaneous and phytohemagglutinin (PHA) stimulated DNA synthesis of lymphocytes was inhibited by this agent. However, a short pretreatment of the lymphocytes by PHA (10 min, 37°C) abolished the inhibitory effect. The factor has various immunological activities. 1. The factor increased the activity of lytic, 19S IgM type antibodies against sheep red blood cells in vitro. 2. Spontaneous rosette formation of spleen cells with sheep red blood cells was inhibited by the factor, which may suggest that it is released from the plasma membrane of macrophages under the conditions of culture. On the basis of these findings a role of this factor in the regulation of lymphocyte activity is suggested.

ISOLATION AND CHARACTERIZATION OF VINBLASTINE-INDUCED AUTOPHAGIC VACUOLES FROM MOUSE PANCREAS

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Autophagocytosis, a lysosomal process, plays a key role in the cellular degradation of endogenous macromolecules. Its morphology and regulation has been thoroughly investigated, but little is known about the specific properties of autophagic vacuoles (AVs). They undergo rapid changes after formation, and their isolation and subsequent investigation in vitro are problematic due to the high fragility of their membranes. Moreover, the densities of lysosomal elements partly overlap those of mitochondria and microsomes in liver. The few works on AV fractions deal with liver secondary lysosomes, sometimes with their densities modified by ingested exogeneous materials. We used vinblastine to elevate the volume fraction of AVs in mouse pancreas where normally they are scarce. After a very mild homogenization in 0.3 mole/l sucrose, a cell debris including nuclei and apical (zymogen) zones of the cells was pelleted by centrifuging for 10 min at 500 g. The supernatant was used for the separation of AVs from rough surfaced microsomes by 4.15 h centrifugation at 125 000 g through a discontinuous sucrose density gradient. As assessed by electron microscopy, microsomes sedimented through the 1.5 mole/l layer and we were able to collect 3 AV fractions from the 1.0, 1.25 and 1.5 mole/l layers. The specific activity of both acid phosphatase and cathepsin D was found to decrease from the lover to the higher density AV fractions. This was confirmed by electron microscopic acid phosphatase assay of the fractions, too. Therefore, these fractions are likely to include AVs in different stages of their development, and the method is a promising tool for their further characterization.

L-ARGININE IS THE MOST POWERFUL ENDOGENOUS FORMALDEHYDE CARRIER IN HUMAN BLOOD AND URINE

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The spontaneous N^{ε}-methylation and formylation reactions, previously shown to take place between L-lysine and formaldehyde (E. Tyihak, L. Trezl, I. Rusznak, Pharmazie 35 18-21, 1980; L. Trezl, I. Rusznak, E. Tyihak, T. Szarvas, B. Szende, Biochem. J. 214 289-293, 1983) could not be detected between L-arginine and formaldehyde, but hydroxymethylation of L-arginine did occur. The hydroxymethylated compounds were isolated from the reaction mixture and also from human blood and urine (A. Csiba, L. Trezl, E. Vari, G. Teglas, Medical Hypotheses 8 209-212, 1982). The guanidino group of L-arginine reacts very rapidly with formaldehyde. We supposed that in biological systems the rate of this addition reaction must be the highest in the case of the compounds which carry guanidino groups.

We have investigated synthetic peptides containing L-arginine, and other compounds: urea, hydroxy-urea, cisteine, AMP, ATP, aglutamine, nicotineamide, formamide, etc. Thin layer chromatography, radiochemical analysis, ^{13}C-NMR-spectroscopy and UV-spectroscopy were applied for the investigations. It was found that the most rapid formaldehyde addition reaction is that of L-arginine, therefore L-arginine is the most powerful endogenous formaldehyde carrier in human blood and urine.

SOME PROPERTIES OF "ANTIHEMOLYTIC" PEPTIDE FRACTIONS OF TRYPSIN DIGESTED CASEIN

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Dephosphorylated (at pH 10) Merck casein was digested with trypsin (trypsin/casein ratio was 1/30). Three main peptide fractions of digested casein were obtained by Sephadex G-25 chromatography. The two largest peptide fractions (peak I. and II.) exert an inhibitory effect (up to 80 per cent) on complement mediated IgM lysis of sheep red blood cells and they have a stimulatory effect on the rosette forming activity of murine lymphocytes.

Casein kinase was used (at saturating γ-^{32}P-ATP concentration) to obtain ^{32}P labelled casein. The total amount of the label was found in the first two peaks of trypsin digested casein (80 per cent in peak I. and 20 per cent in peak II.).

Trypsin digestion repeated twice with elevated concentrations of the enzyme (trypsin/casein ratio was elevated to 1/20) resulted in the disappearance of u.v. absorption at 280 nm of peak I. and the marked decrease of antihemolytic and rosette forming activity of this fraction. Simultaneously the optical density (at 280 nm) of peak II. and peak III. was elevated. The antihemolytic and rosette forming activity of peak II. increased. However, the ^{32}P activity of peak I. remained unchanged.

The amino acid composition of the first two peaks differed markedly.

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An Improved Method for Purification of Post-proline Endopeptidase from Pig Kidney

(Short Communication)

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(Received August 19, 1983)

After the discovery of post-proline endopeptidase in human uterus (Walter et al., 1971), it was also found in diverse animal tissues and was suggested to participate in the catabolism of proline-containing peptide hormones (cf. Walter et al., 1980). In previous purification methods (Koida, Walter, 1976; Orlowski et al., 1979; Hauzer et al., 1982) the enzyme was precipitated from the tissue homogenate with ammonium sulfate, followed by different chromatographies: ion-exchange, Sephadex G-100, hydroxypatite and affinity chromatography. By introducing acetone fractionation of the homogenate before ammonium sulfate fractionation and ion-exchange chromatography, we elaborated a convenient purification method giving a high yield of post-proline endopeptidase.

Determination of enzyme activity. The activity of post-proline endopeptidase (0.1–1 μM) was spectrofluorimetrically monitored (Knisatschek et al., 1980) with an Opton PM QII spectrofluorimeter equipped with two M4 QIII monochromators and a Servogor recorder. The substrate used was Z-glycylproline 2-naphtylamide (Yoshimoto et al., 1978) (5.8 μM in the cuvette) in 01. M phosphate buffer, pH 8.0, containing 1 mM EDTA and 1 mM cysteine or DTE.

Acetone treatment. All purification steps were performed between 0 and 4 °C unless otherwise stated. Fresh or frozen pig kidney (800 g) free of fat and connective tissues, was homogenized in appropriate portions in a Waring blender for 3 times 30 s allowing 1 min cooling between the runs. The homogenization was performed in 2.4 l of 0.02 M phosphate buffer, pH 6.5, containing 1 mM EDTA and 1 mM cysteine (standard buffer). The homogenate was occasionally stirred for 1 h and centrifuged in a cheese cloth bag by using a laundry centrifuge. The

* On leave from the Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia, in the academic year 1980–1981.

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Table 1

Purification of post-proline endopeptidase

Purification step	Total absorbance at 280 nm	Activity yield (%)	Degree of purification
Homogenate	220 000	100	1
Acetone treatment	—	78	—
Ammonium sulfate precipitation and dialysis	5 200	62	27
DE 32 Cellulose chromatography	430	65	335

cloudy supernatant (2.4 l) was treated in an ice bath with acetone (1.6 l) which had previously been cooled to −15 °C. The organic solvent was added during 10 min with constant stirring. After standing in the ice bath for additional 10 min, the precipitate was removed by centrifugation at 10 000 × *g* for 10 min.

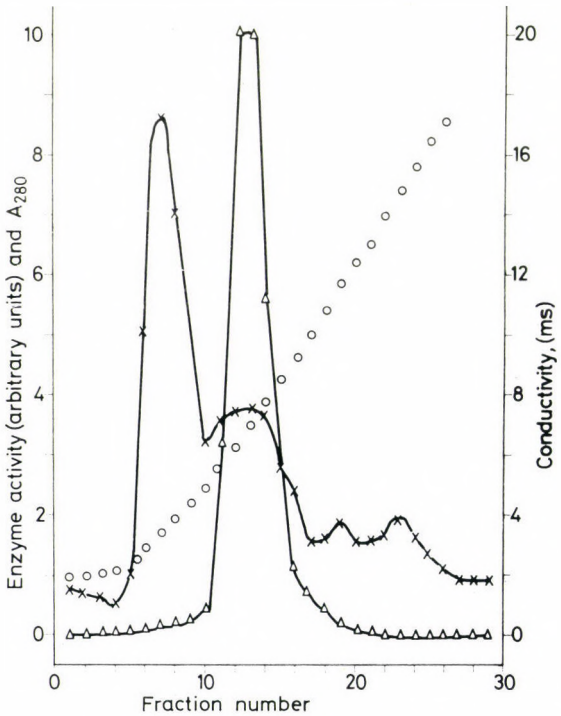

Fig. 1. Chromatography of post-proline endopeptidase on DE 32 cellulose. The activity was measured fluorimetrically, the values are given in arbitrary unit. Fractions of 20 ml were collected. △——△: enzyme activity; ×——×: A_{280}; ○——○: conductivity

Ammonium sulfate precipitation. The supernatant (3.3 l) containing the active enzyme in a 40% acetone solution was treated with solid ammonium sulfate (1100 g). The mixture was vigorously stirred at room temperature for 1 h. After standing for an additional 15 min, two liquid phases separated: the lower contained water saturated with ammonium sulfate to 80%, the upper was aqueous acetone. The precipitated proteins accumulated at the interphase. Most of the liquid phases was carefully removed by an aspirator. The protein was collected by centrifugation. The precipitate, which contained a considerable amount of insoluble protein, was extracted first with 80 ml then with 40 ml of water containing 1 mM EDTA and 1 mM cysteine. The combined supernatants were dialyzed for 24 h against three changes of 5 l of standard buffer diluted 2-fold with 1 mM EDTA and 1 mM cysteine.

Chromatography on DEAE cellulose. The dialyzed solution (about 160 ml) was applied to a Whatman DE 32 cellulose column (3 × 17 cm) equilibrated with the standard buffer. The flow rate was 150 ml/h. The column was washed with 470 ml of standard buffer. This was sufficient to decrease the absorbance at 280 nm close to zero. The enzyme was then eluted with a linear sodium chloride gradient (0–0.25 M) in standard buffer (2 × 300 ml) at a flow rate of 90 ml/h. The combined active fractions (Fig. 1) were concentrated to about 10 ml in an Amicon ultrafiltration cell, Model 202, by using a PM 30 membrane. The concentrated solution was diluted in the cell with 100 ml of water containing 1 mM

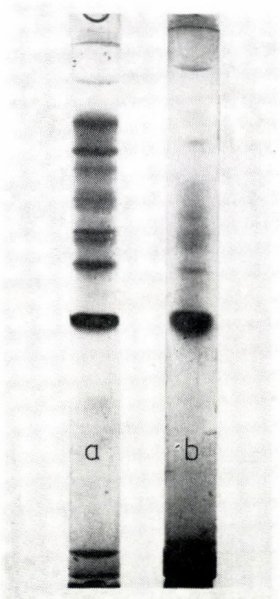

Fig. 2. Gel-electrophoretic patterns of post-proline cleaving endopeptidase before (a) and after (b) chromatography on DE 32 cellulose. The protein bands migrated from the top towards the positive pole. The impurities after chromatography are less than 5 percent

EDTA and 1 mM cysteine and concentrated again to 15–20 ml. The resulting solution was freeze-dried.

Properties of the enzyme. The above procedure provided a relatively pure enzyme in a high activity yield of 65% (Table 1) compared with the 18–25% obtained previously (Koida, Walter, 1976; Orlowski et al., 1979; Hauzer et al., 1982). The purity of the enzyme was examined by polyacrylamide gel-electrophoresis (Davis, 1964) which showed one major protein band (Fig. 2). However, we do not consider that the enzyme is homogeneous because the specific activities in the fractions of the activity peak were not constant (the ratios of the activity to protein concentration were the same only for the two top points of the activity peak as seen from Fig. 1) as it frequently occurs with proteinases.

In agreement with previous data (Orlowski et al., 1979; Walter et al., 1980), the relative molecular mass of post-proline endopeptidase was 67 000 as we tested by sodium dodecyl sulfate polyacrylamide gel-electrophoresis in a vertical slab gel apparatus (4–28% polyacrylamide gradient), and 70 000 when estimated by pore gradient gel-electrophoresis under non-denaturing conditions according to Margolish and Kenrick (1968). This indicates that the enzyme is not composed of subunits.

In comparison with previous methods, the acetone treatment employed rendered it possible to obtain a high yield of post-proline endopeptidase. This may be explained by our finding that post-proline endopeptidase is readily soluble in 40% acetone, which removes a substantial amount of other proteins. The treatment gives rise to a clear supernatant after centrifugation even at low *g* values which is a great advantage when large scale preparations are to be carried out.

Acknowledgement

Thanks are due to Ms S. Szofcsák and Ms J. Fejes for their devoted technical assistance.

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Fluorescence Polarization Analysis of Antigen Determinant Peptides of Human Fibrinogen

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(Received November 30, 1982)

Fluorescamine-labelled human fibrinogen was degraded with trypsin. Using fluorescence detection, the degradation products were fractionated by Sephadex G 50 gel filtration. Fragments of 3000–10 000 molecular weight were isolated for the fluorescence polarization studies.

Specific antifibrinogen antibody increased the polarization of the isolated low molecular weight fragments. These results suggest that even the smallest proteolytic fragments of fibrinogen contain antigen determinant sequences. The fluorescence polarization method offers a sensitive and valuable approach for the analysis of the small antigenic peptides of human fibrinogen.

Introduction

The molecular weights and primary sequences of plasmic or tryptic degradation products of fibrinogen identified as X, Y, D, E are well known and recently the primary sequence of the intact molecule has been established (Mihályi et al., 1976; Doolittle, 1981). However, fewer data are available on the end-products of prolonged digestion of fibrinogen including the possible immunological reactivity of degradation products smaller than fragment E.

The characterization and quantitative determination of these smaller peptides with the widely used immunoassays as immundiffusion, latex agglutination, haemagglutination inhibition immunoassay or different clumping tests are hardly possible (Marder et al., 1971; Smith, Ts'ao, 1973; Hedner, 1976). At the same time the fluorescence polarization (FP) method gives direct insight into the molecular size alterations of proteins (Weber, 1953; Pasby, 1971) and has been found to be applicable to studying antigen-antibody reactions (Dandliker et al., 1964; Spencer et al., 1973; Urios et al., 1978; Kobayashi et al., 1979). The fact that the FP technique is more sensitive when the antigen is small (Pasby, 1971; Spencer et al., 1973) encouraged us to apply the FP technique for the analysis of low molecular weight fibrinogen degradation products.

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In this paper we show that fluorescamine labelled fibrinogen can be attacked by proteolytic enzymes and the rate of digestion can be easily followed by FP analysis. It will also be shown that some of the isolated small tryptic fragments of fibrinogen (molecular weight less than 10 000) act as antigenic determinants as revealed by fluorescence polarization measurements upon addition of the specific antifibrinogen antibodies to the isolated peptides.

Materials and methods

Human fibrinogen (Calbiochem, 90% clottable) was conjugated with fluorescamine (Fluram, La Roche) in 0.2 M H_3BO_3/NaOH buffer, pH 9.0, at 1 : 50 fibrinogen/dye molar ratio. Fluram was dissolved in analytical grade acetone and 1 volume of this solution was added to 3 volumes of protein in borate buffer with vigorous stirring (Udenfried et al., 1972). After staining, the fibrinogen-Fluram complex was subjected to gel filtration. A Pharmacia K15/30 column packed with Sephadex G 50 gel equilibrated with 0.05 M Tris (HCl, 0.1 M NaCl) [pH 8.0] buffer was applied at a flow rate of 1 ml/min. The material eluting in the void volume (from 15 ml to 20 ml) was collected and used for the further experiments.

Clottability test, tryptic digestion and FP measurements were carried out in the Tris/HCl buffer described above. The clottability of stained fibrinogen was checked by the addition of a small amount of thrombin (Topostasine, La Roche) to the fibrinogen preparation.

The fibrinogen-Fluram complex was digested with bovine trypsin (Miles) at 37 °C for different periods of time. In each case 3 mg of trypsin was added to 10 mg of fibrinogen and the proteolysis was stopped by the addition of 6 mg Soybean trypsin inhibitor (Serva, Heidelberg). After treatment with the inhibitor, the FP of the digested samples was determined. In order to check the degree of digestion with another method and also to isolate the low molecular weight fragments, the samples were chromatographed again on Sephadex G 50 gel as described above. Fractions were collected with respect to the elution maxima and the FP of the fractions was determined.

Antifibrin(ogen) antibodies were raised in rabbits. Human fibrin was prepared from pooled citrate plasma by the addition of thrombin and $CaCl_2$. The clot was cut to small pieces, extensively washed in 0.15 M NaCl and sonicated (Braunsonic 300 S) to a homogenous suspension. After protein determination, 10 mg of sonicated human fibrin without Freund adjuvant was injected subcutaneously into rabbits four times at two-week intervals. Antiserum and control serum were gained by bleeding of immunized and nonimmunized rabbits, respectively.

The immunologic activity of the antisera was tested by counterimmunoelectrophoresis method using unstained and stained fibrinogen as antigens. Control and antisera were added to the chromatographically isolated peptide fraction of

digested fibrinogen-Fluram in a final dilution of 1 : 100. There was no remarkable difference in protein content and viscosity between control and antifibrinogen sera. After a short incubation period, the reaction was followed by fluorescence polarization measurements.

The fluorescence and fluorescence polarization measurements were done with a Hitachi Perkin Elmer 203 fluorescence spectrophotometer at room temperature. The excitation was at 395 nm and the emission was analyzed at 480 nm.

In the gel filtration experiments, the elution was monitored by fluorescence detection. In order to do this, we connected the chromatographic column to quartz flow cell built in the fluorimeter.

For polarization measurements the photometer was equipped with two UV transmitting rotatable Polacoat polarization filters (MB105 and PB105) according to Chen and Bowman (1965). Steady state polarization (p) was calculated as

$$p = \frac{I_{vv} - GI_{vh}}{I_{vv} + GI_{vh}}, \quad \text{where} \quad G = \frac{I_{hv}}{I_{hh}}$$

I_{vv} and I_{vh} are the fluorescence intensities measured at vertical excitation filter setting with vertical and horizontal emission polarizers, respectively. G is a correction factor obtained with the incident beam horizontally polarized (Chen, Bowman, 1965). In those experiments in which control and antisera were added to the isolated peptide fragments of fibrinogen, the FP of the samples was determined both before and after the addition of the sera. Polarization values are given as the means of three measurements (the precision of the measure is ± 0.002 polarization units).

Results

Fibrinogen was easily soluble in the mild alkaline (pH 9.0) borate buffer used for fluorescent labelling. The treatment of the protein samples with Fluram dissolved in acetone caused no precipitation at all. The reaction was complete within 30 seconds and the fluorescence of the complex was stable at least 2 days at 4 °C. Clottability of the fibrinogen-Fluram was tested after gel filtration of the complex. There was no significant difference in the thrombin-induced clot formation between the unstained and stained fibrinogen molecules.

Tryptic digestion decreased the FP of the fibrinogen-Fluram complex (Fig. 1). The change in p was very rapid in the first 20 minutes of trypsin treatment. After 1 hour cleavage, we got a much slower decrease in polarization. The degree of fibrinogen degradation was followed not only be FP analysis but also the digested samples were chromatographed on Sephadex G 50 gel (Fig. 2). Undigested fibrinogen was eluted in the void volume without any detectable amount of low molecular weight fragments. The digested samples had characteristic elution patterns. Irrespective of the extent of trypsin treatment the chromatograms showed three elution maxima in identical positions. The relative

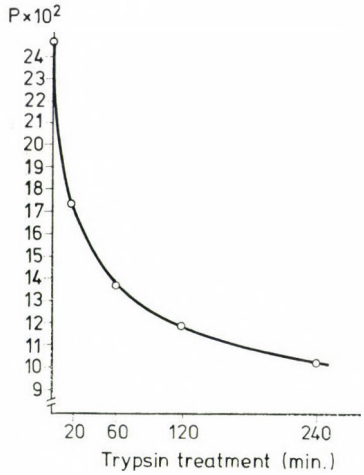

Fig. 1. Effect of trypsin treatment on the fluorescence polarization of fibrinogen–Fluram complex

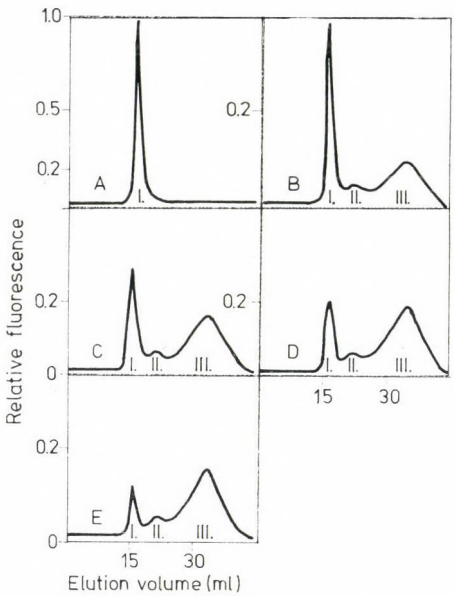

Fig. 2. Sephadex G 50 gel filtration of undigested and trypsin-digested fibrinogen–Fluram complex. (A) Without trypsin, (B) 20 min, (C) 60 min, (D) 120 min, (E) 240 min trypsin treatment

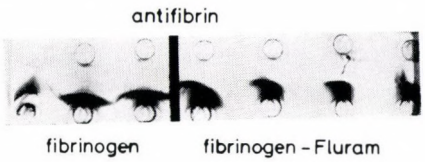

Fig. 3. Counterimmuno-electrophoresis of unstained and Fluram-stained fibrinogen

Table 1

Antifibrinogen-induced changes in the fluorescence polarization of the 3000–10 000 molecular weight proteolytic fragments of fibrinogen separated by Sephadex G 50 gel filtration

Samples	Polarization (p)
Peak III No serum added	0.086
Peak III Control serum added	0.160
Peak III Antifibrinogen serum added	0.287

quantity of the material under peak I (molecules larger than 30 000 daltons) gradually decreased as the proteolytic fragmentation proceeded. The small fragments (peaks II and III) appeared at an early stage of digestion and the amount of peak III was increasing. The FP values of the degraded fibrinogen molecules (see Fig. 1) were in good correlation with the elution patterns obtained by gel filtration. The polarization of isolated peak I remained unchanged in every case ($p = 0.249$) while that of peak III was low ($p = 0.086$).

Counterimmuno-electrophoresis with antifibrin (ogen) serum showed that antibodies gave precipitation with unstained and stained human fibrinogen as well (Fig. 3).

When control and antifibrinogen sera were added to the isolated low molecular weight fragments (peak III) the FP increased in both cases. The increase was much higher if antiserum was added (Table 1).

Discussion

The main purpose of our fluorescence polarization study was to test whether or not antigen determinant sequences are present even in the smallest proteolytic fragments of human fibrinogen. The selection of fluorescamine for labelling of

fibrinogen molecules was based upon two different observations. 1. The complete amino acid sequence of fibrinogen indicated a fairly uniform distribution of the more than 100 lysine residues along the peptide chains of fibrinogen. Consequently, we were logically expecting fluorescent labels on the majority of small fragments of the digested and previously stained fibrinogen molecule. 2. Proteins conjugated with fluorescamine were found to maintain their biological functions including their antigen-antibody reactivity (Sung et al., 1978).

Our fibrinogen-Fluram preparation was clottable after the addition of thrombin. This result indicates that the incorporation of dye molecules did not inhibit the specific proteolytic action of thrombin and the biological activity of fibrinogen was not destroyed. The unchanged immunological character of stained fibrinogen was confirmed by counterimmuno-electrophoresis. The antifibrinogen antibody gave the same precipitation with stained fibrinogen as with the unstained molecules.

In order to get small fibrinogen degradation products the labelled molecules were degraded with trypsin. Trypsin could split the stained molecules therefore we supposed that most of the cleavage sites of trypsin on fibrinogen were not blocked by the bound fluorescamine molecules.

The initial rapid and the late slow decrease in polarization (see Fig. 1) can be related to the kinetics of trypsin action. Nevertheless, the moderate decrease of p during prolonged digestion may be partly due to a self-degradation of the trypsin molecules in the alkaline buffer used in the experiments.

Since we were interested in the small fibrinogen degradation products the trypsin-treated samples were chromatographed on Sephadex G 50 gel. In this gel fragment E, which is the smallest among the high molecular weight degradation products, is eluted in the void volume (Fig. 2). In the fractionation range, two well separated peaks were found. The molecular weight of the peptides in peak III is about 3–10 000 as judged by the positions of the elution maxima. The fluorescence polarization of peak III is $p = 0.086$ which is much lower than that of the digest applied to the column ($p = 0.249$).

When sera of non-immunized (control) rabbits or antifibrinogen rabbit sera were added to the isolated peptides (peak III) an increase in the polarization of fluorescence was found (see Table 1). Although the protein concentration and viscosity of the sera were the same, a much higher increase in p was observed with the antiserum. This finding suggests that some of the small peptides in peak III bind specifically to the antifibrinogen antibodies present in the antiserum. At the same time, nonspecific molecular interactions between the stained small fragments of fibrinogen and proteins of the rabbit serum must be assumed. At this moment, we do not know those serum proteins which interact "non specifically" with the small fibrinogen degradation products. We are also unable to answer the question, whether this interaction occurs only under experimental conditions or it has a biological significance in the metabolism of fibrinogen.

The increase in fluorescence polarization after specific antigen-antibody reaction indicates that the FP technique is suitable for the detection of antigen

determinant sequences of human fibrinogen. As it has been reported recently, some of these or even smaller fibrinogen-derived peptides may play an important role in the regulation of the coagulation system (Hermans, McDonagh, 1982; Kudryk et al., 1982). Further analysis and quantitative determination of the antigenic peptides originating from fibrinogen in pathological serum and urine by the fluorescence polarization method may be of great importance in different diseases accompanied by coagulation disorders.

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Interaction of GGCC Sequences of DNA with Anticancer Dianhydrogalacticol, Detected by Inhibition of Restriction Enzyme BspI

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Interaction of DNA with 1,2-5,6-dianhydro-galactitol (DAG, NSC 132 313), a bifunctional alkylating agent used in cancer therapy was studied.

Treatment of λ phage DNA with DAG *in vitro* protected some of the specific cleavage sites against the restriction enzyme BspI. The extent of protection depended on the concentration and time of DAG treatment but complete protection was not observed. Since the inactivation of the enzyme by DAG was excluded experimentally, the protection can be attributed to the binding of DAG to GGCC sequences of DNA. These experiments support the finding that guanine is the target of DNA alkylation by DAG (Institóris and Tamás, 1980).

DAG treatment *in vitro* induced single strand breaks on DNA and this effect was also found to be concentration and time dependent.

Introduction

1,2-5,6 dianhydro-galactitol is a bifunctional alkylating agent shown to inhibit markedly tumor growth (Németh et al., 1972) and used in cancer therapy (Eckhardt et al., 1977). The inhibition of DNA synthesis was found in various types of tumour cells (Hidvégi et al., 1976) and interstrand crosslinks were suggested to be responsible for the action (Ötvös, Elekes, 1975; Institóris, Fox, 1978). Interaction of DAG with DNA may be related to the marked antitumour effect but the techniques available were not reliable to specify the sequence of DNA involved in the interaction.

Restriction endonucleases have proved to be very useful in studying specific sequences of DNA. These enzymes were used in studies of alterations of DNA structure by alkylation (Gronenborg, Messing, 1978; Bird, Southern, 1978; Bird, 1978) and DNA-drug adducts (Nosikov et al., 1976; Kelman, Buchbinder, 1978).

Abbreviation: DAG; 1,2-5,6 dianhydro-galactitiol, or NSC 132 313, or 1,6 diepoxy-dulcitol, or 1,2-5,6 dianhydro-dulcitol, DAD.

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In this study, efforts have been made to localize the binding of DAG on DNA by restriction enzymes, using λ phage DNA, treated with DAG *in vitro*.

Materials and methods

Preparation of the BspI enzyme was performed according to methods described (Koncz et al., 1978).

Preparation of DNA. Bacteriophage DNA λ cI857 Sam7 was prepared from purified phage particles (Allet et al., 1973). RSF 1010 plasmid DNA was prepared by the SDS-salt and polyethylene-glycol precipitation method (Guerry et al., 1974; Humphreys et al., 1975). DNAs were stored in 0.01 M Tris-HCl (pH 7.4) at 4 °C.

DAG treatment of DNA. DAG was obtained from Chinoin Pharmaceutical and Chemical Works, Budapest. The reaction mixtures contained: 4–6 μg phage DNA, or 0.1 μg plasmid DNA in 0.05 M Tris-HCl buffer (pH 7.4). DAG was dissolved immediately before use in distilled water and applied at concentrations as indicated in the text. Reaction mixtures were incubated at 37 °C in 300 μl plastic reaction tubes in a total volume of 60 μl.

Digestion with restriction enzymes. DNA was taken directly from the DAG reaction mixtures, without removal of DAG. Two-three μg phage DNA was treated with DAG, then digested with ten lambda units of BspI enzyme. The digestion was made in 0.03 M Tris-HCl (pH 8.0), 0.02 M $MgCl_2$, 0.05 M NaCl 0.01 M 2-mercaptoethanol. Incubation was performed at 37 °C for 90 min, and digestion was terminated by the addition of 20 mM EDTA (final conc.).

Electrophoresis was performed in 0.036 M Tris-HCl (pH 7.5), 0.03 M NaH_2PO_4, 0.001 M EDTA (Loening, 1969) in 0.6 × 22 cm cylindrical agarose gels. Gel concentration was 0.8% for the separation of RSF 1010 plasmid DNA or 1.2% for that of BspI digestion products. Electrophoresis was performed with 1 mA per tube for 14 h or 4 mA per tube for 5 h. Gels were stained with ethidium bromide and photographed (Sharp et al., 1973).

Results

Addition of DAG to DNA immediately before the addition of BspI enzyme did not cause any difference in the restriction pattern, as shown in Fig. 1. Since neither freshly dissolved DAG nor its possible decomposition product(s) inactivated the enzyme under the given conditions, it was unnecessary to remove the DAG from the digestion mixtures. There was also no smear or charge effects caused by binding of DAG to DNA which might alter the electrophoresis pattern.

DNA was treated with DAG for different periods of time up to 48 hours and then digested with BspI enzyme. The effects of DAG treatment during 8 h incubation are shown in Fig. 2. At low concentrations of DAG (Fig. 2 *i* to *o*) complete digestion of DNA was reached. Increasing the concentration of DAG,

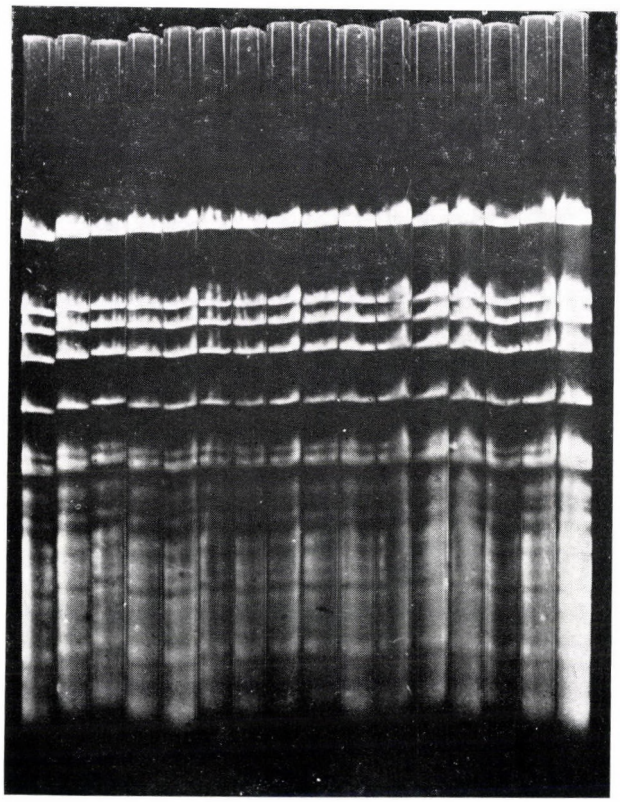

Fig. 1. Inactivation of BspI enzyme by DAG treatment. DAG was added to λ phage DNA immediately prior to the addition of enzyme. Concentrations of DAG in mM: *a*, 500; *b*, 250; *c*, 125; *d*, 62; *e*, 31; *f*, 15.6; *g*, 7.8; *h*, 3.9; *i*, 1.9; *j*, 0.97; *k*, 0.48; *l*, 0.24; *m*, 0.12; *n*, 0.06; *o*, 0.03; *p*, control without DAG

specific "extra bands" appeared, clearly visible in the high molecular weight region of the gels (Fig. 2 *b* to *h*). The appearance of these specific fragments can be interpreted as the result of incomplete digestion, in consequence of the binding of DAG to the GGCC restriction sites, resulting in partial protection against the enzyme. The other effect of the drug was the appearance of a smear which became prominent at the higher concentrations (Fig. 2 *a* to *c*), and made the specific fragments hardly detectable. The smear indicated nonspecific fragmentation of DNA. Experiments will be presented below regarding the nature of fragmentation.

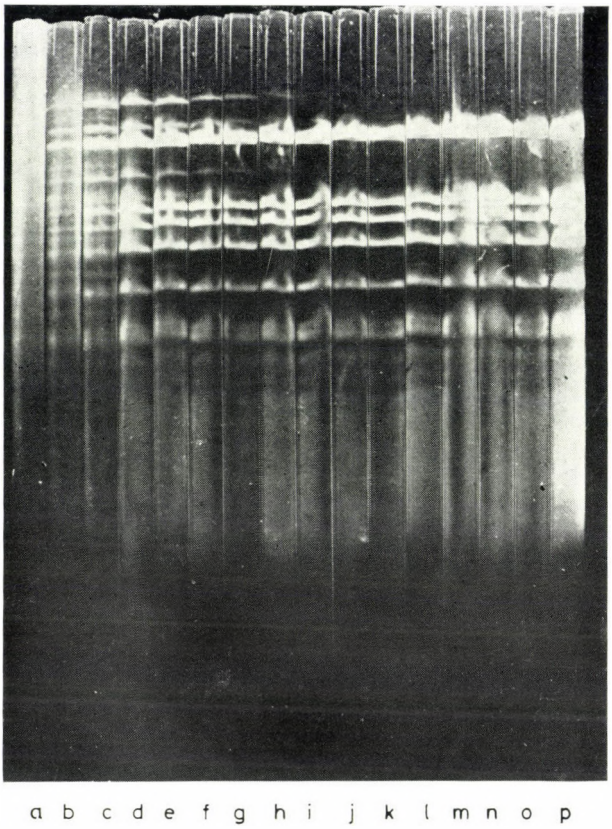

Fig. 2. Appearance of partially digested products in a BspI digestion pattern of λ phage DNA treated with DAG for 8 h. Concentrations of DAG in mM: *a*, 500; *b*, 250; *c*, 125; *d*, 62; *e*, 31; *f*, 15.6; *g*, 7.8; *h*, 3.9; *i*, 1.9; *j*, 0.97; *k*, 0.48; *l*, 0.24; *m*, 0.12; *n*, 0.06; *o*, 0.03; *p*, control without DAG. Extra bands can be seen on gels *b* to *h* and unspecific smears in gels *a* to *c*

During treatment for 48 h, the nonspecific fragmentation proceeded even further (Fig. 3 *a* to *e*), and because of the heavy smear the specific "protected" fragments were seen only at lower concentrations (Fig. 3 *f* to *i*).

Two other enzymes were included into these experiments, which also have G and C nucleotides in their recognition sequences: AluI (AG/CT) and SmaI (CCC/GGG). At any concentration of DAG, the digestion pattern did not differ from the untreated control, *i.e.* no extra bands appeared on the gels (data not shown).

The nature of the nonspecific fragmentation of DNA with the drug was examined by treating plasmid DNA *in vitro*. The two forms of untreated plasmid

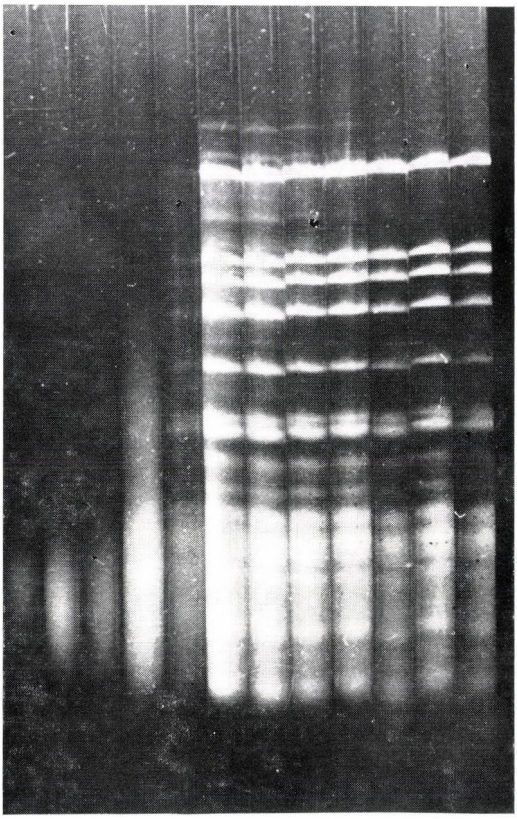

Fig. 3. Effect of 48 h DAG treatment on the BspI digestion pattern of λ phage DNA. Concentrations of DAG in mM: *a*, 500; *b*, 250; *c*, 125; *d*, 62; *e*, 31; *f*, 15.6; *g*, 7.8; *h*, 3.9; *i*, 1.9; *j*, 0.97; *k*, 0.48; *l*, control without DAG. Extra bands can be seen on gels *e* to *i*

DNA are shown on Fig. 4A, the open circles (OC) at the top and the covalently closed circles (CCC) below. On treatment with 0.5 M DAG for different periods of time (Fig. 4A *b* to *e*) the intensity of the CCC band gradually decreased and disappeared completely after 60 min treatment (Fig. 4A *f*). The conversion of the CCC form of the plasmid to OC form indicate the occurrence of single strand breaks on DNA. The lack of the linear form of the plasmid on the same gels suggests that double stranded breaks did not occur.

Similar results were obtained by treatment of the plasmid DNA with different concentrations of the drug for 6 hours (Fig. 4B *a* to *f*). On these gels the linear form of the plasmid also appeared (Fig. 4B *a* to *d*) at high drug concentra-

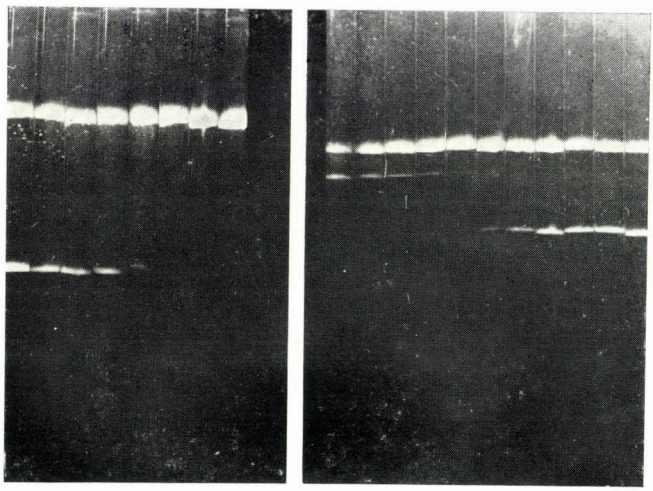

Fig. 4. Strand scission effect of DAG on plasmid DNA. A: RSF 1010 plasmid DNA was treated with 0.5 M DAG for *b*, 5 min; *c*, 10 min; *d*, 20 min; *e*, 40 min; *f*, 60 min; *g*, 90 min; *h*, 120 min; *a*, control without DAG. B: Plasmid DNA was treated for 6 hours, DAG concentrations in mM: *a*, 500; *b*, 250; *c*, 125; *d*, 62; *e*, 31; *f*, 15.6; *g*, 7.8; *h*, 3.9; *i*, 1.9; *j*, 0.48; *k*, control without DAG

tions. The data in Fig. 4B also demonstrate that upon treatment with DAG the CCC form was first converted to open circles by strand scission (Fig. 4B *a* to *f*) and then, as a result of a second single-strand break, the OC form became linear.

Discussion

There are several reports on the use of restriction enzymes in the study of structural alterations of DNA. Actinomycin D and distamycin A were shown by this approach to form adducts with specific sequences of DNA, inhibiting the activity of certain restriction enzymes (Nosikov et al., 1976). Base alkylation (Gronenborn, Messing, 1978; Bird, 1978; Bird, Southern, 1978) and the effect of cis-platinum (Kelman, Buchbinder, 1978) on DNA, has also been investigated by restriction enzymes.

The experiments presented here showed that treatment of DNA with DAG *in vitro* led to the protection some of the GGCC restriction sites against the enzyme BspI. Since the enzyme itself was not inactivated under the conditions applied (Fig. 1), the protection could be attributed to the binding of DAG to GGCC sequences of DNA.

The protection of restriction sites was found to be partial even at the highest concentration of the drug applied here. No quantitative interpretation of the extent of protection was possible, because a great number of the BspI fragments were not resolved on the gels.

The protection of GGCC sequences and the lack of it of AGCT and CCCGGG sequences against AluI and SmaI restriction enzymes, respectively, could be explained by the difference in the number of recognition sites of these enzymes in the phage DNA. Since BspI cuts the phage DNA more frequently than the AluI and SmaI enzymes, the probability that DAG binds to a recognition site is higher with BspI than in the case of the other enzymes. The differences of the flanking regions adjacent to the recognition sites might also be decisive. Nevertheless, these experiments suggest that DAG binds to guanine or to cytosine resulting in the protection of some of the restriction sites, but the binding of the drug to adenine and to thymine bases can not be ruled out. In the *in vitro* reaction of DAG with calf thymus DNA three chemically identified guanine derivatives but no alkylated adenine derivatives were found (Institóris, Tamás, 1980). Therefore, our observations support the finding that guanine is the target of alkylation caused by DAG *in vitro*.

Experiments with plasmid DNA proved that DAG caused single-strand breaks in DNA (Fig. 4). Double-strand breaks were not detected. Due to the fragmentation, we could not examine the DNA for the presence of interstrand crosslinks, which were found to occur upon DAG treatment both *in vitro* (Ötvös, Elekes, 1975) and *in vivo* (Institóris, Fox, 1978).

The strand scission effect caused by the drug *in vitro* was not detected in earlier experiments (Ötvös, Elekes, 1975; Institóris, Fox, 1978). In our experiments, however, strand scission seemed to be the most prominent effect of DAG *in vitro*. The relationship between the protection of GGCC sequences and the single-strand breaks is not clear the protection, however, seemed to occur prior to the strand scission reached a detectable level. Further experiments are needed to clarify whether the two effects occur at the same or at different sites of DNA.

Acknowledgements

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Cloning and Characterization of a Novikoff Hepatoma Ribosomal DNA-fragment Containing the Initiation Site of Transcription

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A 10.3 kilobase EcoRI fragment of Novikoff hepatoma rat ascites tumor ribosomal gene was cloned in pBR322 vector. The cloned fragment contained part of the 18S RNA gene, and about 8 kilobases of the 5' spacer region.

A restriction map was constructed by cleavage of the fragment with BamHI, HindIII, KpnI, PstI, SstI and XhoI enzymes. A putative transcription initiation site for the 45S pre-ribosomal RNA was localized by Pl nuclease protection mapping at a distance of about 130 base pairs 5' to the HindIII site on the restriction map.

Both the restriction map and the position of the initiation site of transcription were almost identical to those of the corresponding rat ribosomal DNA fragments.

Introduction

Analysis of ribosomal and pre-ribosomal RNAs of mouse (Kominami, Muramatsu, 1977), rat (Quagliarotti et al., 1970; Hidvégi et al., 1971) and human (Tiollais et al., 1971) cells demonstrated a marked length and/or 5' terminal heterogeneity of the supposed primary ribosomal RNA transcripts. The heterogeneity was thought to be the result of either the occurrence of transcription initiation at multiple sites or of very rapid processing of the primary transcript, initiated at a single site.

Only a single transcription initiation site was found by single strand specific nuclease protection mapping and by *in vitro* transcription on cloned mouse (Bach et al., 1981a; Grummt, 1981; Miller, Sollner-Webb, 1981; Mishima et al., 1981), rat (Financsek et al., 1982a), and human (Financsek et al., 1982b; Miesfeld, Arnheim, 1982) ribosomal genes.

On the other hand, there is conclusive evidence for the presence of at least two transcription starting points on E. coli ribosomal RNA genes (deBoer et al., 1979; Gilbert et al., 1979; Young, Steitz, 1979).

Abbreviations: rDNA, ribosomal DNA; Pipes, 1,4-Piperazinediethanesulfonic acid; bp, base pairs; kb, kilobases.

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Promoter reduplications in Xenopus laevis (Rungger, Crippa, 1977; Moss, Birnstiel, 1979; Rungger et al., 1979; Maden et al., 1982), X. borealis and X. clivii (Bach et al., 1981b) ribosomal RNA genes were found by *in vitro* and *in vivo* experiments. In the three mammals, although the presence of a single initiation site was clearly demonstrated, there was no conclusive evidence against the presence of additional transcription starting points. It is very difficult to isolate from most mammalian cells, intact, unprocessed primary ribosomal RNA transcripts, because of the high level of endogeneous RNAse activity. In rapidly growing Novikoff hepatoma cells, however, the endogeneous RNAse activity is low, therefore one should be able to isolate intact primary transcripts, even if some of them may represent only a minor component of the total ribosomal RNA transcription.

As a first step toward the understanding of ribosomal gene transcription in Novikoff hepatoma cells we cloned and characterized the part of the Novikoff ribosomal gene that contains the transcription initiation region.

Materials and methods

Materials: Restriction endonucleases, T4 polynucleotide kinase, bacterial alkaline phosphatase, T4 DNA ligase were purchased from Bethesda Research Laboratories. P1 nuclease was obtained from P-L Biochemicals. All enzymes were used according to the suppliers' recommendations.

[γ ^{32}P] ATP was obtained from the Radiochemical Centre, Amersham (PB 10 218) or from the Biological Research Center, Szeged.

All other chemicals were analytical grade commercial products.

Preparation of RNAs: 18S ribosomal and 45S pre-ribosomal RNAs were prepared from nucleoli of Novikoff hepatoma cells as described (Muramatsu, Onishi, 1978; Hidvégi et al., 1971).

P1 protection mapping: The method of Berk and Sharp was followed (Berk, Sharp, 1977) with little modifications (Financsek et al., 1982b). Hybridization of 45S RNA to 5' end labelled DNA was performed in 70% formamide, 0.4 M NaCl, 0.04 M Pipes-NaOH (pH 6.8), 1 mM EDTA at 56 °C for 3 hours, after heat treatment at 90 °C for 3 min. The reaction mixtures were diluted tenfold into cold buffer (0.3 M NaCl, 0.05 M sodium acetate [pH 5.3], 0.1 mM $ZnSO_4$, 10 μg/ml denatured E. coli DNA) and digested at 50 °C for 1 hour with the amount of P1 enzyme indicated in the legend to Fig. 4. The samples were analyzed in 5% polyacrylamide-urea sequencing type gels (Maxam, Gilbert, 1977).

Cloning of the 10.3 kb EcoRI rDNA fragment: Novikoff DNA was digested with EcoRI enzyme and 10–12 kb fragments were recovered from agarose gel (Tabak, Flavell, 1978). The fragments were ligated to pBR322 DNA and after transformation (Enea et al., 1975) the colonies were screened with ^{32}P 18S RNA (Grunstein, Hogness, 1975). Several positive colonies were isolated, and tested

for the presence of 18S and 28S RNA coding sequences. One of those which contained only 18S RNA coding sequence, designated pNH10, was studied further.

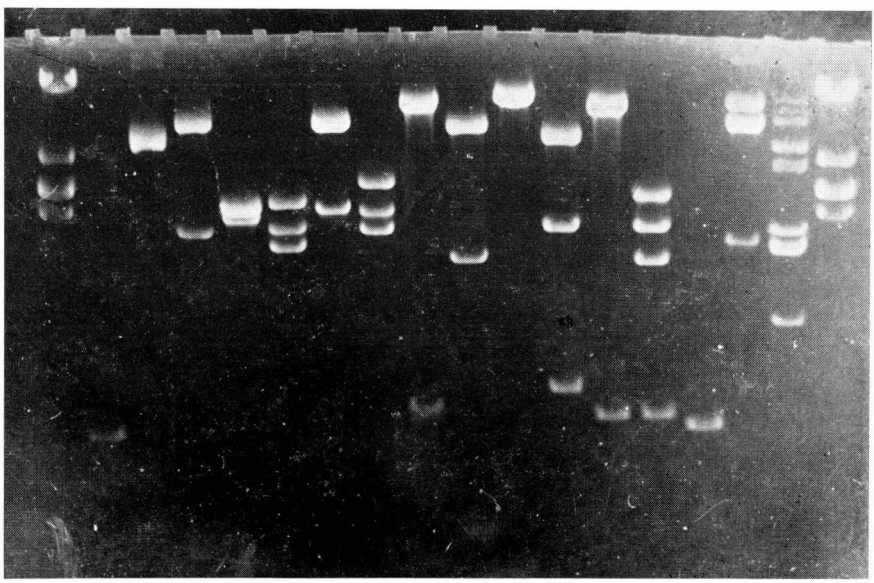
Results

Restriction mapping of pNH10 recombinant DNA

Recombinant plasmid DNA was digested in a series of reaction mixtures with the enzymes indicated below. It is known that the EcoRI, BamHI, HindIII and PstI enzymes have one cleavage site in the vector DNA (Sutcliffe, 1978), the KpnI, SstI and XhoI enzymes do not cleave the vector.

Figure 1, lane 4 shows the vector and the inserted DNA after EcoRI digestion.

The BamHI enzyme (lane 5) cut the recombinant at three sites, two of the generated fragments were almost identical in length. There were two HindIII sites (lane 7), two PstI sites (lane 9), one SstI site (lane 11), two XhoI sites (lane 13) and two KpnI sites (lane 16) on the recombinant DNA. The KpnI digestion was not complete (Figs 1 and 2, lanes 16 and 17).



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Fig. 1. Digestion of pNH10 plasmid with restriction enzymes. Lane 4: EcoRI, lane 5: BamHI, lane 6: EcoRI/BamHI, lane 7: HindIII, lane 8: EcoRI/HindIII, lane 9: PstI, lane 10: EcoRI/PstI, lane 11: SstI, lane 12: EcoRI/SstI, lane 13: XhoI, lane 14: EcoRI/XhoI, lane 16: KpnI (partial), lane 17: EcoRI/KpnI (partial). Lane 3: undigested pNH10 plasmid. Lanes 1 and 18: λ DNA EcoRI fragments, lanes 2 and 15: pBR322 HinfI fragments (only the 1631 bp long fragments are shown). 1% agarose gel

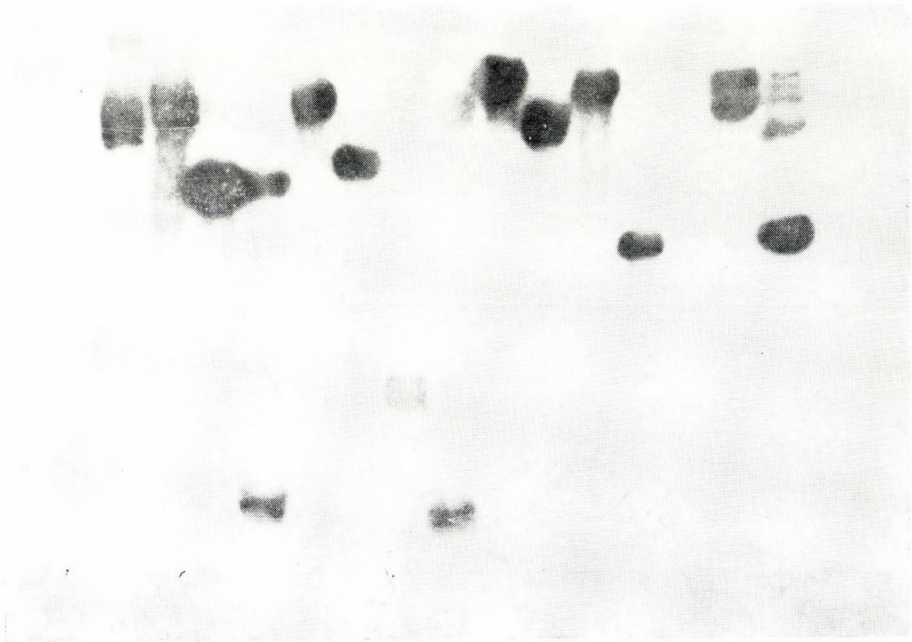
The lengths of the digestion products were determined using EcoRI-digested lambda DNA (lanes 1 and 18) and HinfI-digested pBR322 DNA (lanes 2 and 15) as markers.

The position of cleavage sites and the orientation of the inserted fragment in the vector was determined by Southern blotting (Southern, 1975). The DNA fragments generated by single or double digestion of the pNH10 plasmid with different enzymes (Fig. 1) were transferred to nitrocellulose filter and hybridized to ^{32}P 18S ribosomal RNA. The autoradiogram is shown in Fig. 2.

The 18S RNA hybridized to the undigested plasmid (lane 3) and to the 10.3 kb EcoRI fragment (lane 4).

There was a strong hybridization to the EcoRI/BamHI 1 kb fragment and to the 5 kb BamHI/BamHI fragment (lane 6), indicating that one BamHI site is in the 18S coding region, 1 kb from the EcoRI site, and the other is located 5 kb from the first BamHI site. The 5.7 kb EcoRI/HindIII fragment (lane 8) contained the 18S coding region, evidenced by the strong hybridization.

The orientation of the inserted fragment in the recombinant was determined by PstI digestion. The position of PstI site on the vector is 748 bp from the EcoRI



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Fig. 2. Hybridization of 18S RNA to pNH10 restriction fragments. The DNA fragments seen in Fig. 1 were transferred to nitrocellulose filter and hybridized to 18S RNA in 50% formamide, 0.75 M NaCl, 0.04 M Pipes-NaOH (pH 6.8) 1 mM EDTA 20 μg/ml cold yeast RNA at 37 °C for 16 hours. The autoradiogram of the filter is shown. The numbering of the samples is the same as in Fig. 1

site. The digestion of pNH10 with PstI (Fig. 1, lane 9) yielded a 1.7 kb fragment, giving strong hybridization (Fig. 2, lane 9), which was shortened by 750 nucleotides on EcoRI/PstI digestion (Figs 1 and 2, lane 10). Therefore, the orientation of the insert should be: vector PstI site – 18S coding region – external transcribed spacer (ETS) – non-transcribed spacer (NTS) – vector HindIII, BamHI, SalI sites.

There was no hybridization to the 2 kb EcoRI/SstI fragment (Figs 1 and 2, lane 12) only to the 8 kb one, so the 2 kb EcoRI/SstI fragment was located at the opposite end of the insert than the 18S gene.

Hybridization to the 3.6 kb EcoRI/XhoI (Figs 1 and 2, lane 14), and a lack of it to the 1.7 kb XhoI/XhoI fragment (Figs 1 and 2, lanes 13 and 14) allowed us to determine the order of EcoRI/XhoI fragments unambigously. The 3.8 kb EcoRI/KpnI fragment hybridized to 18S RNA (lane 17) and the 3.9 kb KpnI/KpnI fragment gave no hybridization (lane 16) indicating that the latter is located centrally. From the data in Figs 1 and 2 the cleavage map shown in Fig. 3 was constructed. The relative position of the cleavage sites was confirmed by digestion

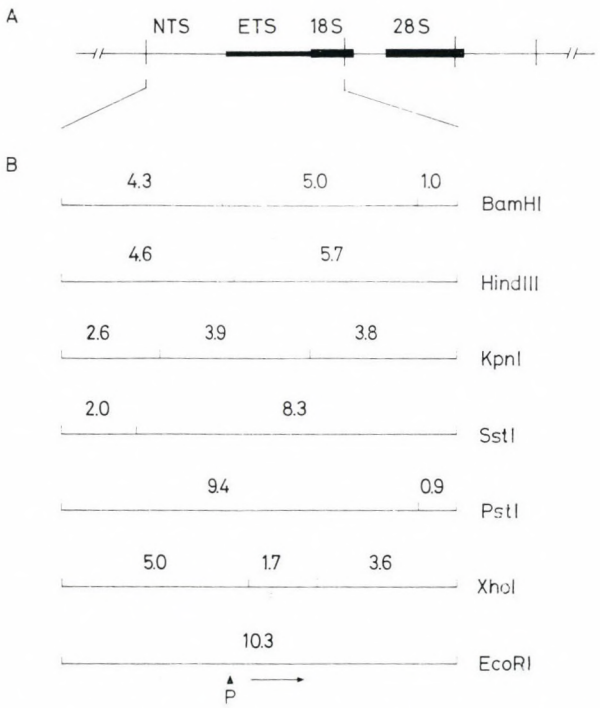


Fig. 3. Restriction map of the pNH10 plasmid. A: The rDNA repeat of Novikoff hepatoma. The EcoRI sites are indicated by the vertical lines. 18S = 18S RNA gene, 28S = 28S RNA gene, ETS = external transcribed spacer, NTS = non-transcribed spacer. B: The cleavage sites of restriction enzymes. On the bottom line, the position of transcription initiation site (P) and the direction of transcription are indicated by the arrows

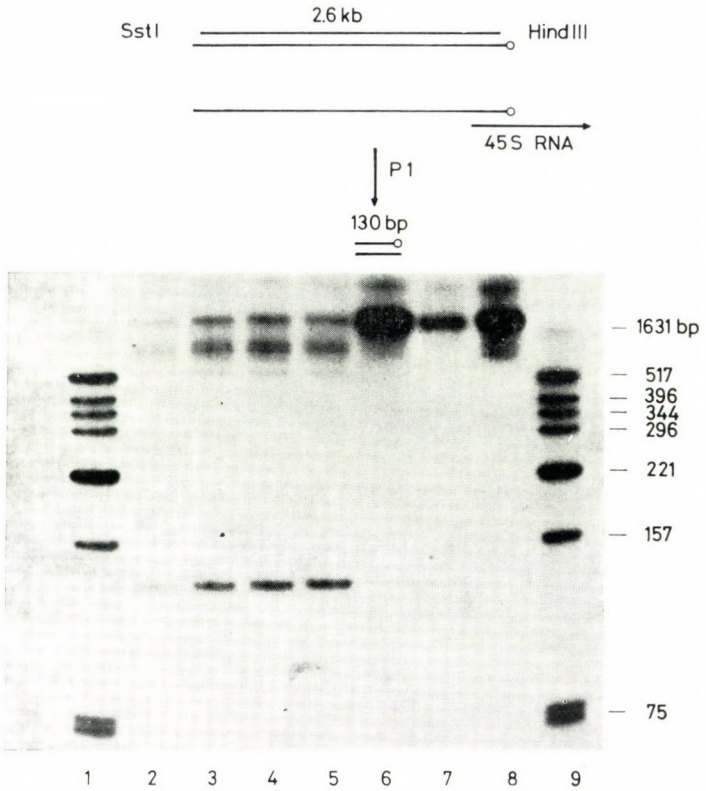


Fig. 4. P1 nuclease protection mapping. The 2.6 kb HindIII/SstI fragment, 5' end labeled on the coding strand was hybridized to 45S RNA as described in Methods, and treated with increasing amounts of P1 nuclease; Lane 2: 53 μg/ml, lane 3: 26 μg/ml, lane 4: 13 μg/ml, lane 5: 7 μg/ml. Lane 6: DNA was hybridized to 45S RNA, not treated with P1. Lane 7: DNA was hybridized to yeast RNA and treated with P1 (53 μg/ml). Lane 8: DNA control. Lanes 1 and 9: pBR322 HinfI fragments. The lengths of fragments are given in base pairs

of the pNH10 plasmid with all of the possible combinations of the enzymes (data not shown).

Determination of the transcription initiation site by P1 protection mapping: The transcription initiation site for 45S pre-ribosomal RNA in a rat ribosomal gene was localized at 127 nucleotides 5' from the HindIII site (Financsek et al., 1982a). Since the restriction map of the rat (Fuke et al., 1981; Financsek et al., 1982a; Rothblum et al., 1982) and Novikoff ribosomal genes was found to be very similar, we performed single strand specific nuclease protection experiments starting from the HindIII site.

The 4.6 kb EcoRI/HindIII fragment was 5' end labelled with ^{32}P, cut by SstI enzyme, and the 2.6 kb HindIII/SstI fragment (5' end labelled on the coding strand) was recovered from agarose gel. Hybridization to 45S RNA was per-

formed as described in Methods, and the hybrids were treated with different amounts of P1 nuclease (Fig. 4, lanes 2 to 5).

As hybridization control, yeast RNA was mock hybridized to the DNA and treated with P1 (lane 7). Also 45S RNA was hybridized to the DNA fragment but was not treated with P1 nuclease (lane 6). DNA control was also run in the gel (lane 8). Marker DNA (lanes 1 and 9) was HinfI-digested pBR322.

Protected DNA bands about 130 bp long can be seen in those samples (lanes 2 to 5) which were hybridized to 45S RNA and treated with P1. The appearance of this band depended on P1 treatment since without P1 (lane 6) only the renatured DNA band can be seen. There are several weaker bands (lanes 2 to 5) longer than the main product but shorter than the renatured DNA. The presence of these protected fragments will be dealt with in Discussion.

Discussion

EcoRI fragment of Novikoff hepatoma tumour cell ribosomal DNA was cloned in pBR322 vector. The length of the fragment was 10.3 kb, determined by digestion of the recombinant DNA with different restriction enzymes, and measuring the length of the different digestion products (Fig. 1). A restriction map was constructed by cleavage with EcoRI, BamHI, HindIII, KpnI, PstI, SstI and XhoI enzymes, and by Southern hybridization with 18S ribosomal RNA (Figs 1 and 2). The order of the digestion products and the orientation of the insert in the vector was also obtained by these two methods.

The restriction map obtained here was found to be identical to those of rat ribosomal gene fragments (Fuke et al., 1981; Financsek et al., 1982a), except the position of KpnI sites. The lengths of the EcoRI/KpnI fragments were similar to those of the rat gene (Rothblum et al., 1982), the order of the 3.9 kb KpnI/KpnI and the 2.6 kb KpnI/EcoRI fragments, however, was found to be reversed in the Novikoff clone (Fig. 3).

We found a putative transcription initiation site by P1 protection mapping (Fig. 4, lanes 2 to 5) at a distance of about 130 nucleotides 5' from the HindIII site. The exact localization at nucleotide level has not been performed yet, but this site seems to correspond to the transcription initiation site found on a rat gene 127 nucleotides 5' from the HindIII site (Financsek et al., 1982a).

We found several protected DNA bands longer than 130 nucleotides (Fig. 4, lanes 2 to 5) which may indicate the presence of multiple initiation sites in the Novikoff gene. The possibility, however, that these resulted from partial DNA renaturation could not be ruled out. Further experiments are in progress to clarify whether or not the Novikoff ribosomal gene has multiple transcription initiation sites.

Acknowledgement

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Structural Changes in Phosphorylase *b* as Revealed by Proteolysis with Subtilisin BPN'

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The proteolysis of rabbit skeletal muscle phosphorylase *b* was studied with Sepharose 4B bound subtilisin BPN' in the absence and presence of various ligands. The proteolysis was carried out at pH 7.0 and pH 8.5 and was followed by measuring phosphorylase *b* activity and by SDS gel electrophoresis. The effect of ligands proved to be qualitatively the same at both pH values.

It was found that AMP and α-D-glucose-1-phosphate accelerated the inactivation of phosphorylase *b* by subtilisin, two main proteolytic products (Mr 70 000 and 30 000) were formed in the presence of these ligands. IMP and glycogen protected phosphorylase *b* against proteolytic attack. Subtilisin treatment in the presence of D-glucose, caffeine and D-glucose-6-phosphate produced a reproducible increase (about 20%) of phosphorylase *b* activity. This "activation" resulted in an increased V_{max} of phosphorylase *b* though did not alter the subunit size, the aggregation state and the ligand binding capacity of the enzyme.

Introduction

Glycogen phosphorylase (EC 2.4.1.1) occupies a key position in the regulation of glycogen metabolism. Its activity is controlled by reversible covalent modification (phosphorylation-dephosphorylation) and ligand induced allosteric transitions (for recent reviews see: Fletterick, Madsen, 1980; Dombrádi, 1981; Fukui et al., 1982). Structural changes of the enzyme have been characterized by different methods including limited proteolysis with trypsin (Fischer et al., 1959; Graves et al., 1968; Bot et al., 1973; Iwata, Fukui, 1975; Gusev et al., 1979), chymotrypsin (Nolan et al., 1964; Iwata, Fukui, 1975; Carney et al., 1978) and a neutral proteinase (Carney et al., 1978). In previous papers we utilized subtilisin BPN' to reveal structural differences between phosphorylase *b* and *a* (Dombrádi et al., 1983a) and to analyse allosteric transitions in phosphorylase *a* (Domb-

Abbreviations: G-1-P, α-D-glucose-1-phosphate; G-6-P, D-glucose-6-phosphate (anomeric mixture); glucose, D-glucose (anomeric mixture); PMSF, phenylmethyl-sulfonyl-fluoride.

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rádi et al., 1983b; Gergely et al., 1983). In the present communication we report on the effect of ligands on the structure of phosphorylase *b* as studied by the same method.

Materials and methods

Crystalline phosphorylase *b* was prepared from rabbit skeletal muscle and the nucleotide contamination was removed by Norit A treatment (Fischer, Krebs, 1962). Phosphorylase *b* activity was assayed according to Illingworth and Cori (1953). The protein concentration was measured spectrophotometrically at 280 nm, an absorption coefficient of 1.30 $mg^{-1}\ cm^{-2}$ was used (Kastenschmidt et al., 1968). The preparations had a specific activity 40–45 U/mg and proved to be homogenous by SDS gel electrophoresis (Laemmli, 1970).

Subtilisin BPN' (Sigma) was immobilized to CNBr-activated Sepharose 4B (Pharmacia) as described by Raibaud and Goldberg (1973). Immobilized subtilisin was used because of its improved stability.

2 mg/ml phosphorylase *b* was treated with 20 mg/ml subtilisin-Sepharose gel suspension (containing 380 μg/ml immobilized subtilisin BPN') at 30 °C in 40 mM Tris-HCl, 2 mM EDTA, 10 mM β-mercaptoethanol (TEM) buffer at pH 7.0 or pH 8.5 in the absence or in the presence of various effectors. Samples were withdrawn from the reaction mixture, diluted 100-fold in TEM pH 6.8 buffer containing 0.1 mM PMSF and the residual phosphorylase *b* activity was determined. The effectors after dilution and PMSF did not influence phosphorylase *b* activity when tested by the method of Illingworth and Cori (1953). Other samples were diluted in a buffer containing 2% SDS and 0.1 mM PMSF and subjected to SDS gel electrophoresis according to Laemmli (1970). Subtilisin-Sepharose was separated from the samples by centrifugation only when specifically indicated.

Results

Effect of ligands on the digestion of phosphorylase b with subtilisin

The effect of subtilisin treatment on phosphorylase *b* is shown in Fig. 1. In accordance with our earlier findings the activity of phosphorylase *b* is nearly resistant to the protease action at neutral pH, however, it gradually diminishes when the reaction is carried out at pH 8.5 (Dombrádi et al., 1983a). Consequently, the influence of ligands accelerating the reaction can be studied at pH 7.0 while the effect of ligands protecting phosphorylase *b* activity can be analysed at pH 8.5 in a more suitable way.

The effect of ligands is qualitatively the same at pH 7.0 (Fig. 1A) and at the pH optimum (8.5) of subtilisin BPN' (Fig. 1B). AMP, a strong activator of phosphorylase *b*, greatly enhanced the rate of inactivation by subtilisin. In contrast IMP, a weak activator, exerted protective effect against proteolysis. The effect of substrates of phosphorylase was also investigated, G-1-P increased while

glycogen decreased the rate of phosphorylase *b* inactivation by subtilisin. Glucose, caffeine and G-6-P share a common effect on the proteolytic reaction: in the presence of these effectors phosphorylase *b* activity increased by 20–30% upon subtilisin treatment.

The combined effect of selected ligands was also tested at pH 7.0. The rate of phosphorylase *b* inactivation was expressed as a pseudo-first order rate con-

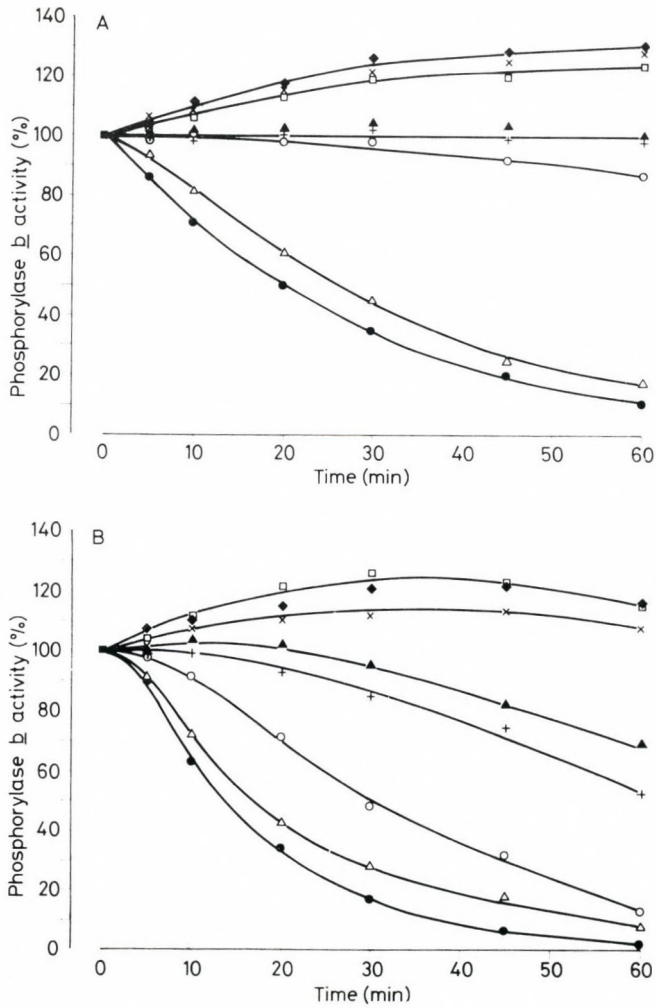

Fig. 1. Changes in phosphorylase *b* activity upon proteolysis with subtilisin-Sepharose at pH 7.0 (A) and pH 8.5 (B). The subtilisin treatment was carried out with no addition (○) or in the presence of the following ligands: 50 mM G-1-P (●); 1 mM AMP (△); 5 mM IMP (▲); 1% glycogen (+); 50 mM glucose (□); 50 mM G-6-P (♦) and 1 mM caffeine (×). The initial activity of phosphorylase *b* (80–90 U/ml) was taken as 100%

Table 1

Effect of ligands on the inactivation of phosphorylase b by subtilisin

Phosphorylase *b* was treated with subtilisin-Sepharose at pH 7.0 and its inactivation was characterized with a pseudo-first order rate constant. The following ligand concentrations were used: AMP, 1 mM; G-1-P, 50 mM; glycogen, 1%. The mean of three experiments is shown (s.d. ± 15%)

Ligand	$k \times 10^3$ (min^{-1})
None	2
AMP	30
G-1-P	39
AMP + G-1-P	47
Glycogen	0
AMP + glycogen	15
G-1-P + glycogen	7

stant (Table 1). The combination of AMP and G-1-P in saturating concentrations resulted in the highest inactivation rate. Glycogen reduced the accelerating effect of AMP or G-1-P on the inactivation process.

Properties of the proteolytic products

As shown in Fig. 2 SDS gel electrophoresis revealed that subtilisin produced an Mr 70 000 fragment from the 100 000 phosphorylase *b* subunit in the absence of effectors. In the presence of AMP the digestion resulted in a significant reduction of protein in the Mr 100 000 band and a massive appearance of Mr 70 000 as well as 30 000 bands. Similar observations were made earlier under somewhat different conditions (Raibaud, Goldberg, 1973; Dombrádi et al., 1983a). The effect of G-1-P on proteolysis proved to be similar to that of AMP according to the SDS gel pattern. When subtilisin treatment was completed in the presence of glucose no obvious change in the native subunit could be detected by SDS gel electrophoresis. There was a minor reduction in the amount of total Coomassie stained material in gels 2 and 4 indicating that the Mr 30 000 fragment was totally or partially degraded in the absence of additions or in the presence of G-1-P, respectively.

The stability of the nicked phosphorylase *b* was compared to the native enzyme in heat inactivation studies (Fig. 3). Native phosphorylase *b* fairly well survived two hours at 45 °C; glucose exerted a slight protective effect on the heat inactivation process. The product of phosphorylase *b* after subtilisin treatment proved to be more instable, it got inactivated at 45 °C within two hours. Glucose protected the proteolytic product against inactivation.

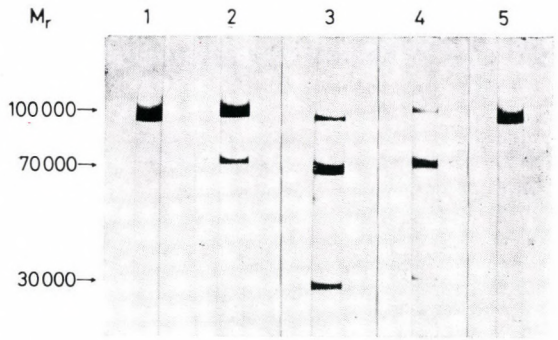

Fig. 2. Proteolytic fragments of phosphorylase *b* as analysed by SDS gel electrophoresis. 8 μg digested protein was loaded on each gel. 10% polyacrylamide gels were run and stained for protein by Coomassie brillant blue R-250. Phosphorylase *b* prior to proteolysis (1) and after 60 min subtilisin treatment at pH 7.0 without effectors (2) or in the presence of 1 mM AMP (3); 50 mM G-1-P (4) and 50 mM glucose (5)

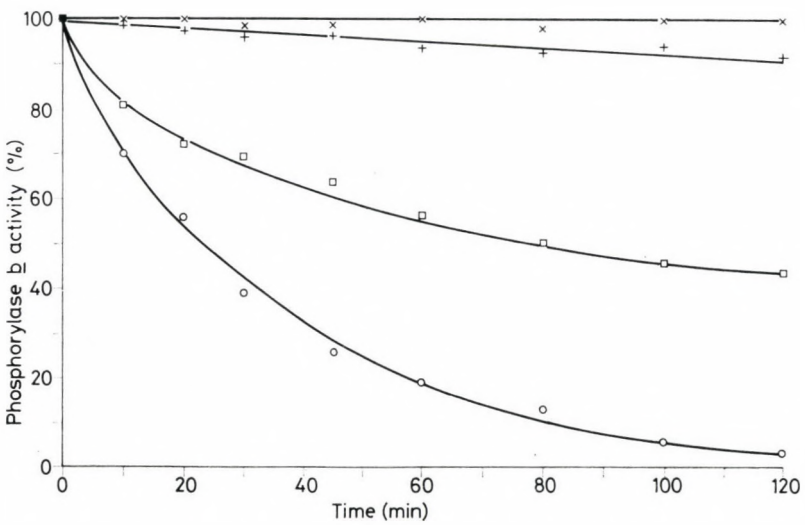

Fig. 3. Heat inactivation of phosphorylase *b* at 45 °C. Phosphorylase *b* was treated with subtilisin at 30 °C, pH 7.0 for 30 min prior to heat inactivation. Proteolysis was arrested by the addition of PMSF and subtilisin-Sepharose was removed from the mixture by centrifugation. The heat inactivation of subtilisin treated phosphorylase *b* in the absence (○) or in the presence (□) of 50 mM glucose was compared to that of the native enzyme in the absence (+) or in the presence (×) of 50 mM glucose at 45 °C. The phosphorylase *b* activities before heat inactivation were taken as 100% (native phosphorylase *b:* 89 U/ml; subtilisin treated phosphorylase *b:* 88 U/ml)

Table 2

Kinetic constants of phosphorylase b treated with subtilisin in the presence of glucose as compared to the untreated (native) enzyme

Phosphorylase *b* treated with subtlisin-Sepharose for 60 min in the presence of 50 mM glucose at 30 °C and pH 7.0, as well as untreated (native) phosphorylase *b* samples were centrifuged at 10 000 *g*, 0 °C for 10 min, and the supernatants were passed through a 0.9 × 60 cm Sepharose CL 4B column. Fractions of the highest phosphorylase *b* activity were collected and analysed using the following ranges of ligand concentrations: G-1-P 1–50 mM; glycogen, 0.005–1%; AMP, 5–200 μM

Constant	Phosphorylase *b*	
	subtilisin treated	untreated
K_M G-1-P	3.3 mM	3.5 mM
K_M glycogen	0.02%	0.03%
K_a AMP	60 μM	50 μM
V_{max}	54 U/mg	45 U/mg

The proteolytic "activation" in the presence of glucose did not alter the aggregation state of phosphorylase *b*, the "activated" and native enzymes eluted from a Sepharose CL 4B column at the same position (data not shown). Neither the K_M for G-1-P and glycogen nor the K_a for AMP of the gel filtrated enzymes differed within the experimental errors, the "activated" phosphorylase *b* merely possesed higher V_{max} than the native one (Table 2).

Discussion

As we pointed out (Dombrádi et al., 1983a) the subtilisin digestion of phosphorylase *b* cannot be treated as exactly as that of phosphorylase *a* (Dombrádi et al., 1983b) since several, up to now unidentified, sites of cleavage have to be taken into consideration besides the ones described for phosphorylase *a* (Titani et al., 1977). However, the present work still warrants a qualitative description of gross structural changes of phosphorylase *b* in response to ligand binding.

Previously we reported that the rise in pH from 7.0 to 8.5 resulted in a significant structural change of the subtilisin sensitive part of phosphorylase *b* (Dombrádi et al., 1983a). Now, we found that the effect of various ligands on proteolysis was qualitatively the same at both pH values (Fig. 1). The two activators of phosphorylase *b*, AMP and IMP, induce different structural changes in the enzyme. Such a difference has already been observed in kinetic (Black, Wang,

1968), calorimetric (Ho, Wang, 1973), fluorescence (Vandenbunder et al., 1976) SH-reactivity and ultracentrifugation (Busby, Radda, 1976; Morange et al., 1976) as well as chemical cross-linking (Hajdu et al., 1979) experiments and can be attributed to the fact that AMP preferentially binds to the nucleotide site of phosphorylase while IMP equally occupies both the nucleotide and nucleoside binding sites (Uhing et al., 1979).

The conformational changes brought about by AMP and G-1-P are recognized by subtilisin to be very similar (Figs 1 and 2). One of the common features is that G-1-P protects the Mr 30 000 fragment from further proteolysis as reported for AMP earlier (Dombrádi et al., 1983a), though the protective effect of G-1-P is not as pronounced as that of AMP (Fig. 2). The Mr 30 000 fragment represents the N-terminal regulatory part of the phosphorylase subunit (Raibaud, Goldberg, 1973) so it is tempting to speculate that G-1-P protects this fragment, because in the absence of AMP it also binds to the nucleotide site of the enzyme as suggested by Sygush et al. (1977) and Kasvinsky et al. (1978) based on X-ray diffraction experiments. Since AMP and G-1-P influence the quaternary structure (Black, Wang, 1970), cross-linking (Hajdu et al., 1979), SH-reactivity (Jókai et al., 1965; Damjanovich, Kleppe, 1966) of phosphorylase *b* and the properties of a spin-label attached to the enzyme (Birkett et al., 1971) in a distinct way, one has to suppose that the similar effect of these two ligands represent only some common structural changes in the protease sensitive part(s) of the molecule rather than similar overall conformational transitions.

AMP and G-1-P together slightly enhance each others effect on proteolysis (Table 1) probably because of their positive heterotropic interaction that has been well documented in kinetic, spectroscopic and chemical modification studies (cf. Graves, Wang, 1972; Busby, Radda, 1976; Hajdu et al., 1979). Glycogen substantially reduced the inactivation rate obtained in the presence of AMP or G-1-P (Table 1) indicating a strong interaction of the glycogen storage site with the AMP and G-1-P binding sites. Analogous results were found when the combined action of glycogen and AMP was studied by calorimetric measurements (Merino et al., 1977) and cross-linking with bifunctional diimidates (Hajdu et al., 1979). As phosphorylase exists *in vivo* in interaction with glycogen in the form of a protein-glycogen complex (Meyer et al., 1970) the above results suggest that conformational changes of phosphorylase studied in the absence of glycogen – though are interesting from a theoretical point of view – presumably do not represent the events occuring under physiological conditions.

Subtilisin treatment decreased the heat stability of phosphorylase *b* even in the case when no appreciable change in phosphorylase *b* activity can be detected during the proteolytic reaction (Fig. 3). It suggests that some nicks are "silent" or they compensate each others effect on the catalytic activity. Glucose protects the nicked phosphorylase *b* from heat inactivation, indicating that glucose can bind to the proteolytic products of phosphorylase *b*. In accordance, binding of glucose to subtilisin treated phosphorylase *a* was demonstrated with fluorescence quenching technique (Gergely et al., 1983).

Our most unexpected result is the reproducible increase in phosphorylase *b* activity when treated with subtilisin in the presence of glucose, caffeine and G-6-P as shown in Fig. 1. The "activation" of phosphorylase *b* seems to be associated with the proteolytic removal of small N- and/or C-terminal peptide(s) from the enzyme because no detectable change in the subunit size was found by SDS gel electrophoresis (Fig. 2). The "activation" did not influence the binding of substrate or activator (AMP) to phosphorylase *b*, however, increased the V_{max} of the enzyme (Table 2). One can assume that subtilisin created a more flexible phosphorylase structure capable of faster activity-related fluctuations (Welch et al., 1982). "Activation" can be achieved only when digesting phosphorylase *b* in the presence of glucose, caffeine and G-6-P. These ligands are all inhibitors of phosphorylase *b* and probably favour a conformation in which a specific portion of the polypeptide backbone gets susceptible to subtilisin.

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On the Effect of the Discrete Nature of the Photoelectric Emission in the Thermoluminescence Measurements (Pulse Counting Method)

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The discrete nature of the photoelectric emission cannot be neglected at low reading levels. The effect of A/D conversion after photomultiplication were both calculated and measured. The reading and its standard deviation were considerably different from those expected on the basis of a simple Poisson distribution and this should be taken into account at low reading levels.

The authors describe a simple method which permits the experimental determination of photoelectron/digit conversion factor necessary for the prediction of the variation coefficient of TL measurements.

Introduction

The charge integrating (DC and pulse counting) method is used practically in all commercially available TL readout instruments.

The authors have published a theoretical model (Zaránd, Polgár, 1983a) which permits the calculation of the relative standard deviations of these TL measurements at arbitrary dose levels provided that three constants of the detector-readout system are known. The first constant is dependent on the background subtraction method (Zaránd, Polgár, 1983b), the second one, B is the high-dose performance of the whole system expressed in relative units, and the last one is the photoelectron/dose conversion factor (which is the product of constants photoelectron/digit, k, and digit/dose, k_2). If, however, the signal (background or TL) to be measured is small, the reading and its coefficient of variation (σ_R/R) will be different from those expected on the basis of theory, presumably as a result of analog-to-digital (A/D) conversion.

The purpose of the present paper is twofold: *i.* to establish a method for measuring the photoelectron/digit conversion factor and *ii.* the investigation of the effect of the analog-to-digital conversion on the reading and on its measured coefficient of variation at very low working levels.

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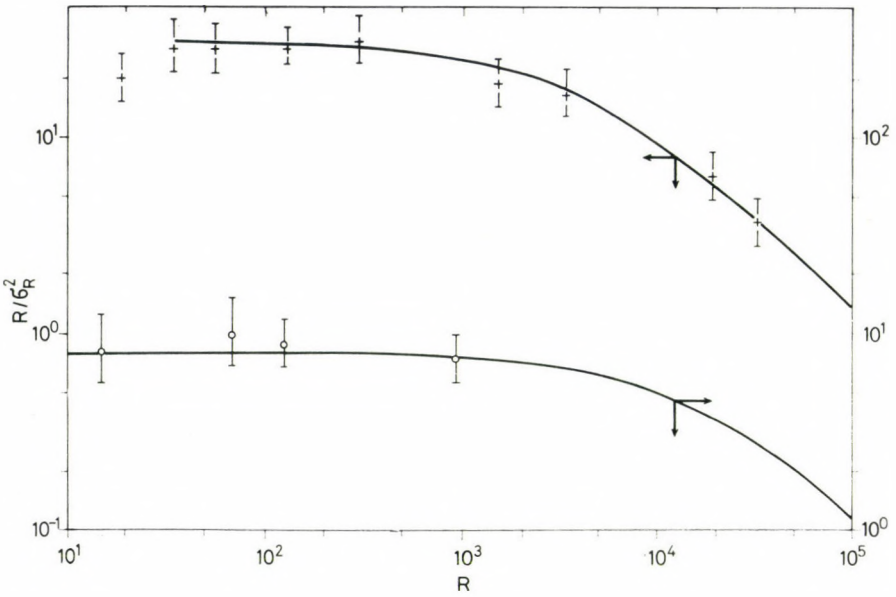

Fig. 1. Experimental determination of the photon per count conversion factor from the light source measurements. HV = 1200 V (upper line) or 1450 V (lower line)

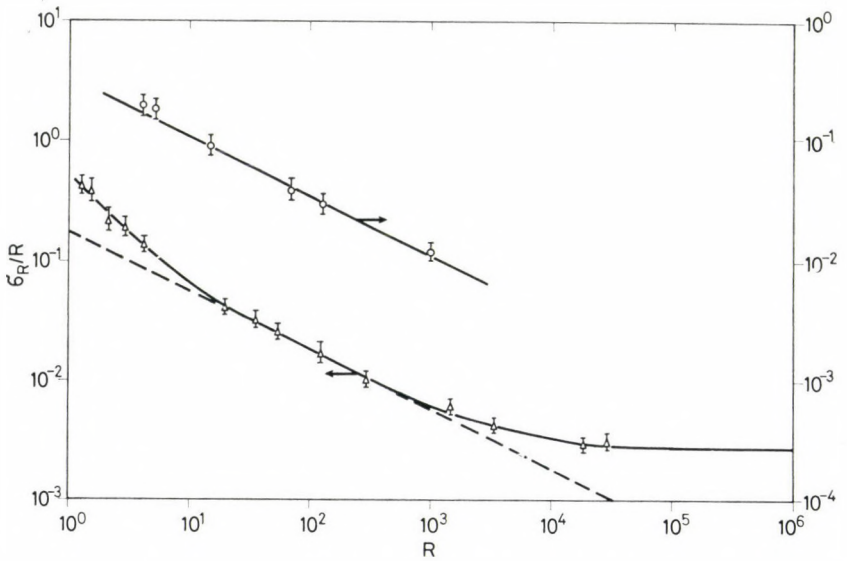

Fig. 2. The coefficient of variation obtained with an external light source vs. reading measured with a TL readout instrument. HV of the PM tube 1200 V (lower line) and 1450 V (upper line). Error bars in all figures represent 95 percent confidence limits of usually 75 or 100 measurements

The measurement of the photoelectron/digit conversion factor

The light emission of a beta-source embedded in a plastic scintillator has a Poissonian distribution. This statistics is preserved in the electron emission of the photocathode (RCA, 1970) of a good photomultiplier (used in TL measurements). The light detection is practically a TL measurement without any non-radiation background. For this case our theory is reduced to

$$\sigma_R/R = \sqrt{\frac{1}{kR} + B^2} \tag{1}$$

if the reading is expressed in digits. In the present experiments $B \approx 0.003$. From Eq. 1. k may be determined with sufficient accuracy if $B^2 \ll 1/Rk$.

We have measured the emission of the light source for 10 s. The light intensity was changed by inserting stepwise exposed X-ray films between the light source and the PM tube (HV = 1200 V). The measurements were repeated with a HV setting of 1450 V. The density, d of the film was determined with a Macbeth–Kollmorgen 502 LB densitometer. k was determined from measurements performed at various density levels and plotted in Fig. 1. It is evident from these measurements that k should be determined in the optimal range but it can be determined experimentally. At low reading levels (below about 5), however, the curve is declined. In this range the measured light intensity differs from $I = I_0 \cdot \exp(-d)$ and the variation coefficient of measurements deviates considerably from the value expected for a Poissonian distribution (the curve should have a slope of -0.5 on the log-log scale, Fig. 2). We assumed that this deviation results from the A/D conversion.

The investigation of the effect of the A/D conversion at low readings

The effect of the analog-to-digital (A/D) conversion may be calculated as follows. Let us assume that all pulses of the PM tube represent the same charge, and one digit at the output of the A/D converter is exactly equal to k pulses on the PM with a total charge of Q. The effect of the A/D conversion on the mean and relative standard deviation of a Poisson distribution can be seen in Fig. 3 (with no charge on the A/D converter at the moment of starting of counting). In practice, however, at the beginning of the measuring cycle the reading is equal to zero but at the same time the charge accumulated in the A/D converter is equal to $x \cdot Q/k$ ($x = 0, \ldots, k-1$) with equal likelihood of $1/k$. The charge due to the signal is added to this value. No charge is lost during continuous A/D conversion process except the fraction at the end of the total counting period. The Poisson distribution of the signal (photoelectrons emerging from the photocathode) was replaced with a Gaussian (parameters n and $\sqrt{n}$) if $100 < n$. The convolution of the two distributions and the distribution obtained as a result

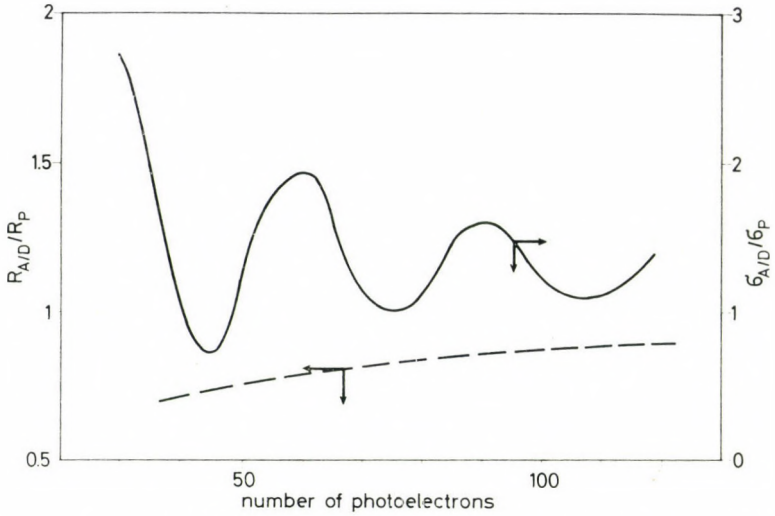

Fig. 3. The effect of the A/D conversion on the mean and on the standard deviation of a Poisson distribution if the charge is equal to zero at the beginning of the counting. R_P and σ_P refer to the Poisson distribution. These values are changed to $R_{A/D}$ and $\sigma_{A/D}$ after conversion. Calculations

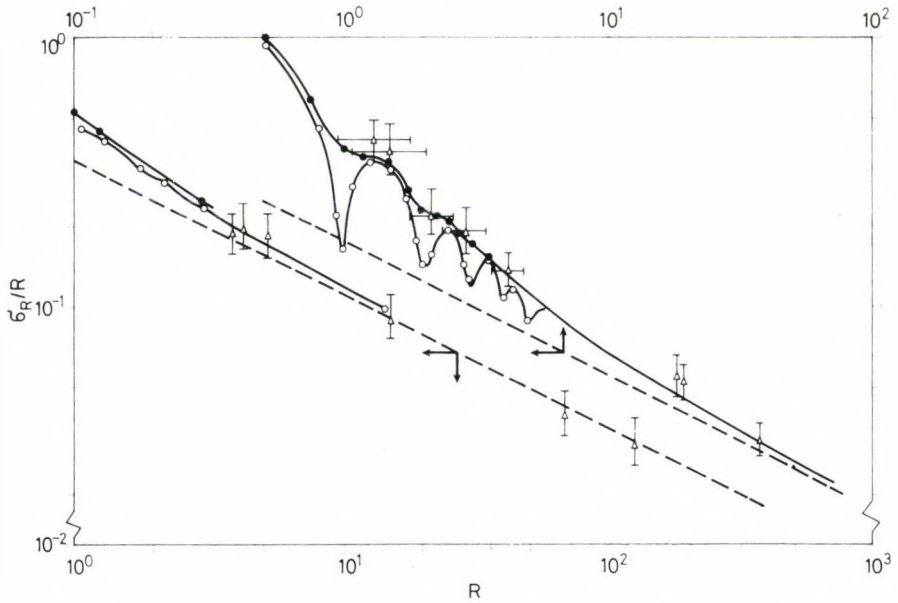

Fig. 4. The effect of A/D conversion on the coefficient of variation. Comparison of measured and calculated values at low reading levels, R (expressed in digits). Dashed line: Poisson distribution without A/D conversion, ●—●: the convolution of a uniform distribution with a Poisson one, ○—○: Poisson distribution after A/D conversion. Upper curves: 1200 V, lower curves: 1450 V

of the conversion was assessed on a PTK-1096 calculator (Hungarian version of Texas TI-59). σ_R/R_{calc} was plotted in Fig. 4 as a function of R_{calc}. The measured values are the same as in Fig. 2. Calculations were made for both k values (8 and 32) obtained in the present measurements. The dashed lines represent the pure Poisson distribution without the effect of A/D conversion.

In mathematical statistics data may be grouped on the basis of the assumption that in a group each value has equal probability. Grouped data should have at least 5–6 groups (Weber, 1961). That means, a group-width, w, should be at least about 1 σ. The correction factor to be applied to the differential distribution $\sqrt{1 + 1/12(w/\sigma)^2}$ is smaller than 1.01 only if $w < 0.5\ \sigma$. The A/D conversion may be considered as a special grouping. Let us have a simple Poisson distribution. At least 5–6 groups may be expected by using a photoelectron/digit conversion factor of 8 if the expected count number is about 15–20. In the case of $k = 32$ we should have at least 80 digits. The conclusion is straightforward. In low dose measurements k should be as small as possible. A small k may be achieved by working with higher HV on the PM tube. The optimal solution in low dose measurements is the "photon counting" with $k = 1$. The variation coefficient of the background of a TL measurement is usually much higher than that of a Poisson distribution and enough groups can only be made if the reading is about 10 digits (Zaránd, Polgár, 1983b).

Conclusion

The photoelectron/digit conversion factor is essential for the prediction of the variation coefficient of TL measurements. This factor may be determined experimentally by the method described in the present paper by using a light source with variable light intensity.

The A/D conversion after photomultiplication changes considerably the reading and its standard deviation at low reading levels. Accordingly, low reading levels should be evaluated very carefully.

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Flow Cytometric Analysis of Viability of Bull Sperm Cells

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Fluorescein diacetate and propidium iodide double fluorescence labeling of animal sperm cells in combination with 55°C treatment for 15 min was used to monitor the viability and heat tolerance of sperm cells by flow cytometry. The applicability of the elaborated test to detect fertility differences has been shown on sperm samples obtained from bulls of known fertility values. According to preliminary examinations the method seems to be suitable to test the fertility of other male animals, too.

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

Introduction

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 semen sample in the veterinary practice has been the microscopic investigation. This type of test provides information about the number of cells in the sample as well as about their morphology and motility, the latter regarded as the main qualifying factor of fertility. However, even this can give only rough information about the potential fertility of the cells. Recently several efforts have been made to improve these simple methods. High speed flow cytometric analysis of sperm cells has been introduced by exploiting the specific properties of acridin orange staining (Evenson et al., 1980). Their method based on metachromasic staining of the chromatin before and after heat treatment of sperm seems to be a more efficient fertility test than the classical microscopic investigation. The great advantage of flow cytometry resides in the accuracy of the evaluation of the test, since a large cell population can be investigated in a reasonable short time. Recently another method of staining sperm cells simultaneously with rhodamine 123 and ethidiumbromide has been elaborated (Evenson et al., 1982). In this case the detection of some lesions of mitochondria can

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be correlated with the potential subfertility. Attempt has been made to determine the viability of bull sperm cells by measuring the fluorescein retaining and propidium iodide (PI) excluding ability of their cytoplasmic membrane (Resli et al., 1983).

The viability of the cells as well as the fertility is influenced by the actual condition of the cell membrane. The principal aim of the present work is to elaborate a quick method for qualifying semen samples by testing the resistance of the membrane bound enzymes (e.g. nonspecific esterases) and the membrane permeability simultaneously against environmental stress. Fluorescence double labeling with fluorescein diacetate (FDA) and PI was applied in combination with 55 °C heat treatment of bull sperm. The measurements were carried out in a Becton–Dickinson FACS-III flow cytometer.

Materials and methods

Bull semen was freshly taken and transported at room temperature to the place of the measurements. Before the measurements stock solution was prepared with phosphate buffered saline (PBS) at pH 6.8. Heat treatment at 55 °C was applied on the stock solution of the sperm cells for 15 min. The heating was terminated by quick addition of 20 °C PBS resulting in a final 200 times dilution (2×10^6 cell/ml). This procedure cooled down the heated samples to 20 °C practically immediately.

Staining procedure: 10^{-6} mol/l FDA (Sigma Chemical Co.) was added to the samples from a stock solution of 10^{-4} mol/l dissolved in acetone. After 3.5 min incubation at room temperature, PI (Sigma Chemical Co.) was added from a previously prepared stock solution (1 mg/ml) to the samples at a final concentration of 40 μg/ml.

Flow cytometric analysis: Immediately after the PI had been added the samples were put into a Becton–Dickinson FACS-III flow cytometer to be analysed. The wavelength of the exciting laser beam was 488 nm and the power output was 400 mW. Fluorescence emissions of the two dyes were detected simultaneously; fluorescence resulting from FDA hydrolysis through a 540 nm interference filter (Melles Griot) with a green sensitive multiplier and PI fluorescence through a 620 nm cut off filter with a red sensitive multiplier. Light scattered by the sperm cells was used to initiate the electronic processing of the fluorescent signals.

Typically 5×10^4 cells were collected in the pulse high analyser at a speed of 1000–1500 cells/s.

Results and discussion

Figure 1 shows the correlated frequency distribution of green and red fluorescence of a diluted bull semen sample labeled with FDA and PI. In the figure each dot represents a cell and the position of the dot is determined by the correlated

green (fluorescein fluorescence increases with the X-axis) and red (PI fluorescence increases with the Y-axis) fluorescence emitted by the cells. Analysing these types of plots the cells may be classified into three subpopulations:

1. Live cells: FDA – having hydrophobic character – is taken up by the cell membrane. The nonspecific esterases hydrolyse FDA into fluorescein and

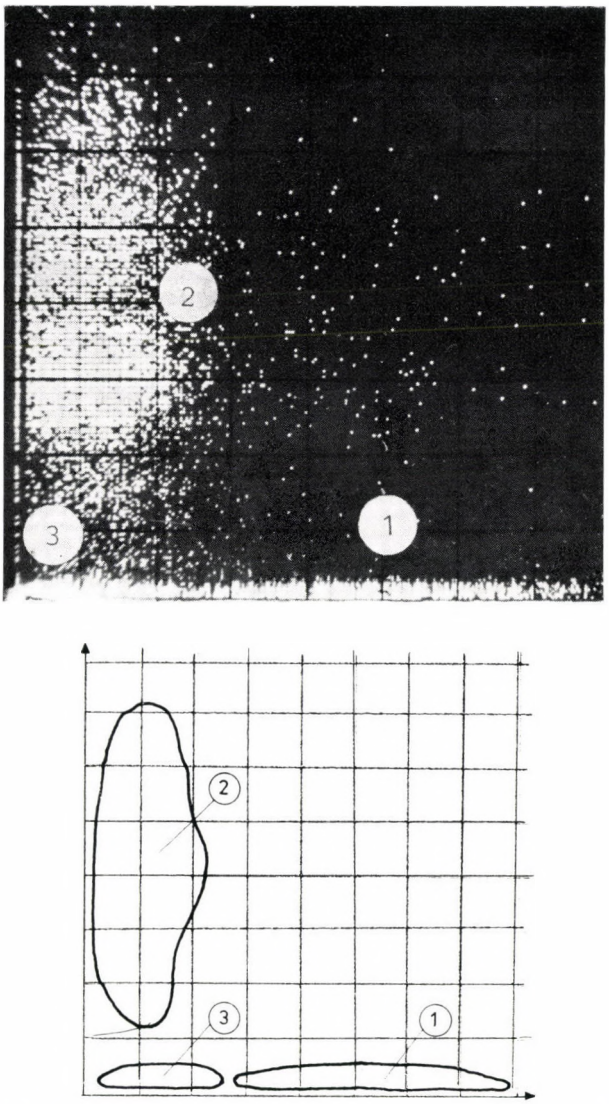

Fig. 1a. Scattergram of bull sperm cells stained with FDA and PI. The coordinate values are proportional to the fluorescence intensities of the fluorescein (X-axis) or PI (Y-axis) content of the cells. b. Three subpopulations are distinguished: 1) Live cells; 2) dead cells; 3) debris

acetic acid residue (Rotman, Papermaster, 1966; Szöllősi et al., 1981). The hydrophylic fluorescein is trapped inside the cells, thus all cells having intact cytoplasmic membranes will show a bright green fluorescence. Since the PI does not penetrate the undamaged membranes, the live cells will not show red fluorescence.

2. Damaged or dead cells: Owing to their damaged membranes these cells are leaky for fluorescein even if their esterases split the FDA. At the same time the PI can penetrate through the damaged membrane and intercalate into the DNA (Szabó et al., 1982). The intercalated PI has no deexcitation through the proton transfer process towards the aqueous environment compared to the situation with dissolved dye. Thus its quantum yield is significantly increased and the damaged or dead cells show an intensive red fluorescence.

3. Debris: The third population, with low intensity fluorescein and PI fluorescence near to the origin in Fig. 1 corresponds to highly damaged cells, or debris and smaller unidentified particles of the seminal fluid. This part of the dot plot can be distinguished from the other regions of interest. We did not make further effort to separate it from the rest of the sample. This was in consonance with our aim to elaborate a quick and versatile method to qualify the semen samples.

Figure 2 shows the fluorescence intensity distribution of a bull semen sample with known fertility (53.5 percent conception rate) for both red and green fluorescence. Part A shows the fluorescein fluorescence of the sample. The left peak with

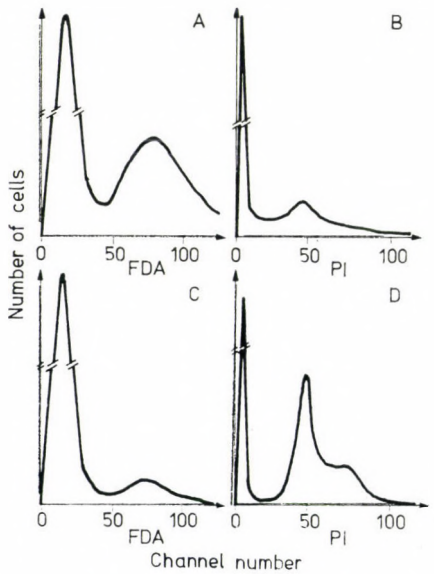

Fig. 2. Fluorescence intensity distributions of a sample with 53.5% conception rate before and after heat treatment. Fluorescein flluorescence before (A) and after (C) 15 min heat treatment. PI fluorescence before (B) and after (D) heat treatment

Table 1

The effect of heat treatment on bull semen samples

Code number of the bull	Percentage of live cells		Conception rate (%)
	Untreated sample (%)	Heat treated sample (15 min, 55°C) (%)	
7633	86	27	53.5
7756	81	19	48.8
7364	75	3	40.7
7867	90	2	40.6

low intensity green fluorescence corresponds to the debris and dead cells, while the second one represents the live cells. Part B of the figure shows the frequency distribution of the intensity of the red, PI fluorescence. Here the first peak represents the live cells and second the damaged or dead ones. The broad shoulder of the second peak indicates that the flattened sperm cells are oriented parallel or perpendicular to the laser beam (Gledhill et al., 1976). The percentage of the live cells in the sample can be calculated from the comparison of the areas under the peak: in Part A and B.

The lower part of the figure displays results of aliquots of the same sample exposed to 55 °C for 15 min before staining. It can be seen that the number of the live cells decreased (C), while the number of the damaged or dead cells increased (D). The percentage of the live cells was calculated as 86 percent of the total number of cells before, and 27 percent after the heat treatment.

Table 1 demonstrates the effect of the heat treatment on the semen samples of different bulls. In the table percentages of live cells before and after heat treatment, together with the conception rates characterizing the fertility of the different bulls are displayed. It can be seen that the initial live cell percentages of the different bulls are in all cases reasonably high and they do not correlate with the conception rates in the Table. If the heat treated samples are compared to the untreated ones a big decrease in the live cell percentages can be observed. Moreover, the heat treated values favourably compare with the conception rates of the respective bulls.

It is important to note that in all cases the semen samples were carefully examined by microscope. Taking into consideration the error of the microscopic motility and cell count determinations, no differences could be found by this method between the different samples. The morphology of the samples from the different bulls was also indistinguishable.

Our method has been applied for qualifying the semen of several bulls with known fertility index. The results showed good agreement with the field tested conception rates of the animals even in those cases where the microscopic examination did not reveal any difference between their semen. Preliminary investigations

have been performed on other animal species (boar, turkey-cock and ram) and the results obtained are similar to the above demonstrated ones. These data support our view that the advantage of the flow cytometric analysis, the double fluorescence labeling in combination with the heat treatment can be successfully applied to measure the potential fertility of male animals.

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Long Time Stability of Purple Membranes from *Halobacterium halobium*

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The fast component of the electrical signal and the light absorption signals at different resolution times (at $\lambda = 408$ nm) in an untreated purple membrane suspension were measured in order to study the stability of purple membrane fragments.

It has been established that there is no change in the rate constants of $L \rightarrow M$ and $M \rightarrow O$ transitions of the photocycle in either the electrical or the light absorption signals of purple membrane suspension kept at 4°C for up to 40 days. Moreover, also the amplitudes of the I (negative) and II (positive) components of the electrical signal remained unchanged during this period.

Introduction

Bacteriorhodopsin (bR), the only protein contained in the purple membrane (PM) of Halobacterium halobium, acts as a light driven proton pump (Lozier et al., 1975; Oesterhelt, Stoeckenius, 1973). Spectroscopically different intermediates included in the light induced reaction cycle of bR have been studied by flash photolysis, modulation excitation spectrophotometry (Slifkin, Caplan, 1975; Sherman, Caplan, 1975; Lozier et al., 1975; Lozier et al., 1976), low-temperature spectroscopy (Stoeckenius, Lozier, 1974; Lozier et al., 1976).

Flash-induced electric signals from bR in the PM have been observed in model systems (Hong, Montal, 1979; Drachev et al., 1978) and PM suspension (Keszthelyi, Ormos, 1980).

It has been already shown that an orientation of PM in suspension can be achieved by applying a low static electric field – 20 V/cm (Keszthelyi, 1980). Oriented untreated PM suspensions were used in order to study the transient electrical signals due to charge movement during the photocycle and a good correlation of the electrical components with the intermediate steps in the photocycle of bR was observed by Keszthelyi (Keszthelyi, Ormos, 1980).

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According to Singh et al. and to Neugebauer et al. (Singh, Caplan, 1980; Neugebauer, Zingsheim, 1978) the marked stability of the structural and functional properties of bR are maintained for months or even years. On the other hand a reduced H^+/M_{412} ratio (the number of protons released per photocycle) in the PM fragments kept at 4 °C for 1 year has been revealed (Govindjee et al., 1982). The authors have shown that the aged sample had the same behaviour as the PM sheets after the removal of the COOH-terminal region of bR by proteolytic cleavage with trypsin and papain. Govindjee et al. (Govindjee et al., 1980), too, have found that the quantum yield of the photocycle depends on the age of the PM sheets and is much higher for fresh preparations. They supposed that the discrepancies in the experimental results obtained in different laboratories may be due to the aging of the sample. In order to avoid such an effect they used 7 to 10 day-old preparations for spectroscopic measurements.

The aim of this study is to provide information about the stability of functional properties of bacteriorhodopsin.

Materials and methods

The PM fragments from Halobacterium Halobium strain ET 1001 were obtained according to the standard procedure (Oesterhelt, Stoeckenius, 1974). The measuring system was the same as in the experiments of Keszthelyi et al. (Keszthelyi, Ormos, 1980).

Electrical signals were measured on an oriented PM suspension using platinized (Pt) electrodes at a distance of 0.8 cm. Opton dye laser (λ = 580 nm, 10 mJ energy, 1 μs duration time), transient recorder (NEO 200 B, product of Central Research Institute for Physics, Budapest, Hungary, 0.1 μs the fastest conversion time) and multichannel analyzer (ICA 70, product of Central Research Institute for Physics, Budapest, Hungary) were used. The electrical signals were amplified by a Keithley 604 differential amplifier. For absorption change measurements a 300 W tungsten lamp, Carl Zeiss (Jena) monochromator, EMI 98620/070 photomultiplier were used.

PM preparations were kept at 4 °C. The measurements were carried out in concentrated aqueous suspensions of PM (concentration = 0.35 mM) in a 0.2 cm cuvette at pH 6.8 up to 40 days. The experiments were performed at 21 °C.

Results and discussion

In this paper we report the dependence of some kinetic parameters on the age of the PM sheets.

Figure 1 shows the protein electric response signal (b) and the light induced absorbance changes at λ= 408 nm (a, c), mesured with different time resolutions.

The measurements were performed at time intervals of 2 days for 40 days.

The time constants of the L → M and M → O transitions were calculated and plotted as a function of the age of the PM suspension (Figures 2 and 3).

The time constants remained unchanged for the whole measuring period. The data obtained from the optical measurements indicate that the kinetics of the M_{412} photointermediate decay and yield remain unaffected during this period of storage of the PM suspension at 4 °C. It is in support of the results of Govindjee et al. (Govindjee et al., 1982) for the M_{412} decay measured in a 1 year-old PM suspension. They have reported that the half time of M_{412} decay is not influenced

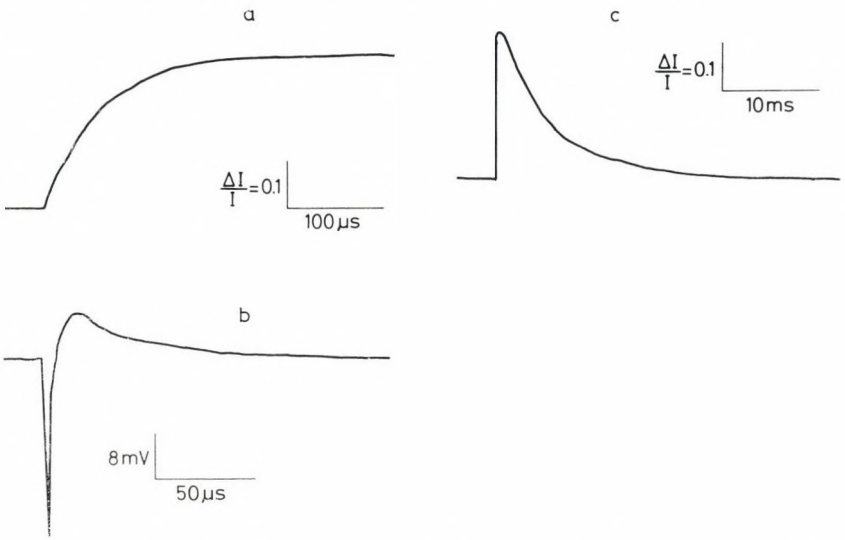

Fig. 1. Light-induced absorption signals (λ = 408 nm) at different resolution times (a, c); fast component of electrical signal (b). Orienting field IOV, measuring resistance 20 kΩ. Optical density of suspension (A = 2.2); pH 6.8; 21°C

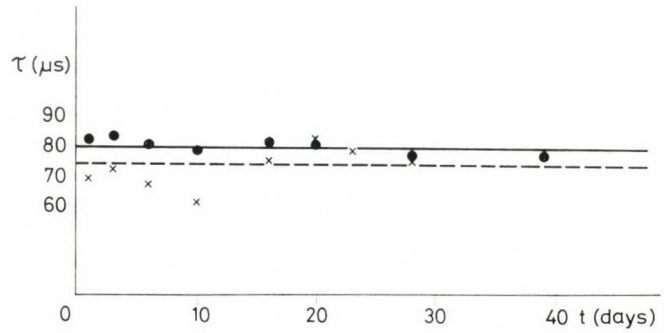

Fig. 2. Time constant of L → M transition vs. the age of the PM fragments. Data from light-induced absorption signals (·) and from the positive component of the fast electrical signals (x)

by prolonged storage of the PM although they observed a change in the number of protons released per photocycle.

The observed stability of proton pumping function of bR obviously reflects the functional and probably structural stability of the purple membranes.

A marked stability of the electrical signal was also observed. The time constant of the L → M transition derived from the positive component of the electrical signal was the same for fresh and one month old membrane fragments. It is clear from Fig. 3 that the time constants of the electrical and the optical signals coincide, which is in support of the data of Keszthelyi et al. (Keszthelyi, Ormos, 1980).

No change in the amplitudes of the positive and negative components of the electrical signals (Fig. 4) after a one-month long storage of the membrane preparations was observed.

In view of these data we claim that, whatever structural and/or functional changes might happen in PM fragments during the storage period, they do not affect the protein response signal and absorbance changes of M_{412} intermediate.

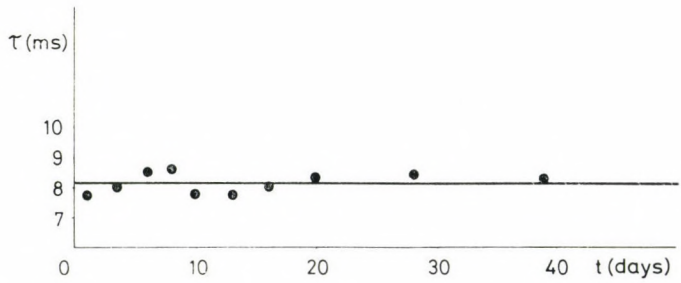

Fig. 3. Time constants of M → O transition vs. the age of the preparations taken from the light absorption signals

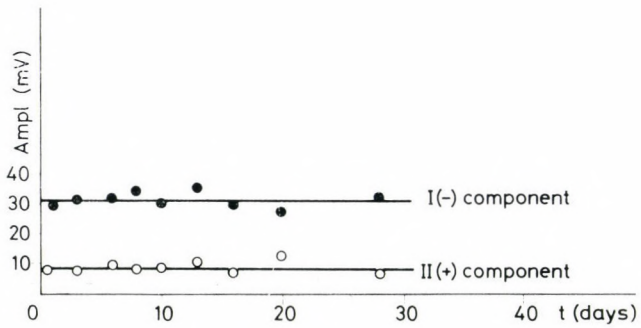

Fig. 4. The dependence of the amplitudes of I negative (·) and II positive (○) component of electrical signal from the ageing of the sample. Measuring resistance 20 kΩ, electric field 10 V

Further investigations are needed to show whether our results are due to a structural stability of bacteriorhodopsin in PM and to study functional properties of bR for a longer period of storage of PM fragments.

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Biological Macromolecules and Liquid Crystals[1]

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1. Nowadays it is already not surprising to speak about the interaction between the research on biological macromolecules and on solids. In the study of biological macromolecules biology applies, in many respects, the *concepts of solid state research:* the study of order-disorder, the relation of structure and properties and that of structure and function. Many times it uses the *experimental and theoretical methods of structural research* applied in solid state physics. Meanwhile, the complexity and high sensitivity of biological systems has given numerous stimuli for solid state physicists to increase the sensitivity and "resolving power" of these methods and to develop their theories in a way which renders them suitable for studying the complex biological systems and for the interpretation of the behaviour of these systems.

It should be particularly emphasized that, in the meantime, the scope of studies on solids has considerably widened and has been extended to partly ordered and even amorphous substances. The *liquid crystals* are of outstanding importance and this is a special field where biology and solid state physics met tematically, too. In isotropic and anisotropic environments various chemical reactions take place differently. Besides, they are influenced by the structure of the anisotropic medium (Usol'tseva, 1983). The cell, as the medium of biochemical reactions, can by no means be considered an isotropic medium. The biological macromolecular systems also behave in several respects as liquid crystalline systems, as *liotropic liquid crystals.*

Liotropic liquid crystalline systems consist of *amphiphilic molecules* (containing a hydrophilic and a hydrophobic part) and of *water* as solvent. Such systems have properties characteristic partly of liquids, partly of crystals. To the former ones belong e.g. the fluidity and the drop forming ability, while to the latter ones the spontaneous optical, electric, magnetic and other anisotropies. It is alo a crystalline property that X-ray scattering curves obtained in liquid

[1] Review held at the 12th National Meeting of the Hungarian Biophysical Society (Budapest, 1983).

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Table 1

Macromolecules studied with respect to liquid crystalline properties

Lipids	Proteins and polypeptides
Lecithins	Trypsin
Kephalins	Haemoglobin
Sphingomyelins	Contractile proteins
Glycolipids	Histones
Phosphoglycerides	Model polypeptides
Monoglycerides	
Cholesterol-esters	
Extracts of biol. membranes	
Nucleic acids	*Polysaccharides*
DNA, RNA	Chitin

crystals exhibit maxima at certain places in the range of small angles which indicates a longer range ordering of the constituents.

2. *Biological macromolecular systems* are usually characterized by the fact that they may exist in a wide range of pH, ionic strength, concentration and temperature and their properties depend considerably on these factors. Of these systems and their subsystems Table 1 summarizes those studied in detail with regard to liquid crystalline properties. The lipids on which numerous results of interest were obtained in Hungary, appear in a particularly high number. A considerable part of these results relate to liquid crystalline systems consisting of lipids and serving to model biological membranes.

A whole series of experimental and theoretical results indicates that the structure and phase conditions and the related transport properties of model membranes are considerably modified by temperature (Marsh et al., 1977), by hydrostatic pressure (Lin, Kay, 1977; De Smedt et al., 1979), by an electric field (Jähnig, 1976; Sugár, Tarján, 1981; Sugár, Neumann, 1984), by changes in the concentration of hydrogen or other ions (Szőgyi et al., 1983) and by the presence of polar, apolar and amphiphilic (Cserháti et al., 1982; Szőgyi et al., 1983; Cserháti, Szőgyi, 1984) compounds. – One practical importance of the results e.g. is that the effectivity of anesthetics and antibiotics influencing the membrane can be predicted on liquid crystalline model systems (Blaskó et al., 1979).

Proteins and polypeptides appear in a lesser number, but as an example it is interesting to mention the liquid crystalline model of the molecular mechanism of muscle contraction (Vazina, 1983).

Nevertheless nucleic acids and polysaccharides as liquid crystalline systems were much less studied.

In the following parts nucleic acids will be dealt with and these systems which, among others, contain also nucleic acids. Nucleic acids exist in the living material usually in interaction with proteins in specially ordered structures. As an

Table 2

Degree of hydration in the case of various T7 DNA preparations

Studied system	V_{H_2O}/V_{DNA}	$n_{H_2O}/n_{base\ pair}$
DNA thin film 20% humidity	0.23	~ 5
DNA thin film 80% humidity	0.86	~ 20
DNA within phage head	1.8	~ 40
DNA in solution	4.5	~100

example it is sufficient to mention the material of the cell nucleus and the chromosomes participating in it. With regard of these, it is only a recent assumption that these structures too, may exhibit liquid crystalline properties (Trifonov, 1983a, b).

Of the nucleic acids and nucleic acid-protein complexes, phage T7 should be mentioned which was studied in several places (Serwer, 1978, 1979; Harpst, 1980; Stroud et al., 1981; Zavriev, Shemyakin, 1981; Mamet–Bradley, Karska-Wysocki, 1982; Krüger et al., 1982). We jointed these studies with our investigations, because the phage T7-nucleoprotein as a simple model system treatable in well defined conditions proved to be of interest in many respects. The base sequence of its nucleic acid and the amino acid sequence of its proteins was only revealed quite recently (Dunn, Studier, 1983). The supramolecular intraphage structure of the main constituents, the role and importance of the nucleic acid–protein interaction contains still numerous unrevealed details.

This summary deals with some aspects of T7-DNA as liquid crystal and related to this, I present our experiences on the damage of the function which indicate the significance of the liquid crystalline structure in certain reactions of biological interest.

3. Table 2 contains different *T7-DNA preparations* giving in each case the water/nucleic acid volume ratio (V_{H_2O}/V_{DNA}) and, calculating from this, the number of water molecules per base pair ($n_{water}/n_{base\ pair}$).

One should consider first the upper row in which data are given on DNA thin film prepared by a special sedimentation method (Fekete et al., 1979). In the determination of the volume ratios the quantity of the solvent was obtained from gravimetric and IR spectroscopic results (Falk, 1964). In similar systems Potaman et al. (1981) directly observed nematic and cholesteric structures. It is justified to assume, that in the case of the T7 DNA thin films also exist in liquid crystalline states. – It is worth to compare these data with the new microcalorimetric results of Mrevlishvili et al. (1982). According to them, in the case of 20 per cent humidity in the environment the quantity of water in our preparation does not reach the 10 water molecules per base pair, i.e. that quantity of structured water based on the GC content of the T7 DNA. It may be assumed that the water

content here is solely structured water. At 80 per cent humidity the water content corresponds to the sum of the structured water and the water in the hydration shell in the DNA molecule.

Considering next the last row of the Table which gives data on disordered (isotropic) T7 DNA solution and comparing the hydration degree of DNA (Serwer, 1975) with the structuredness of water, it is apparent that now there is much "free" water, too, in the system.

The DNA data of the T7 phage head (Tris buffer, 0.1 mol/l NaCl) remained last. These were obtained by small angle X-ray and neutron scattering (Rolbin et al., 1980; Rontó et al., 1983). Considering the volume ratios, this system is between the thin film and the isotropic solution. The 40 water molecules per base pair means that, besides the structured water and the hydration shell, there is inside the phage head a limited quantity of free water as solvent. The *liquid crystalline state can be assumed inside the phage head* as well, this is indicated by the characteristic fine structure also observed on the small angle X-ray scattering curve at 3.5°. This scattering pattern characterizes the arrangement of the DNA threads and may correspond to the liquid crystalline structure inside the phage head (Dembo et al., 1982; Fekete et al., 1982). According to the CD spectrum (Tóth, 1981), the ordering is rather nematic than cholesteric. (This statement agrees with the results of Damaschun et al., 1978 and that of Potaman and Shlyakhtenko, 1982).

In connection with the hydration of the phage head one more remark seem to be necessary: the given value is an average for the whole phage head. Based on recent results (Vainshtein et al., 1982) one may conclude that the hydration is inhomogeneous inside the phage head. Let us consider Fig. 1 showing the electron density distribution within the phage head. The isodensity curves denoted with the number 0.4 belong to the lowest electron density, i.e. to the electron density of the protein coat and the protein core. The DNA has two electron den-

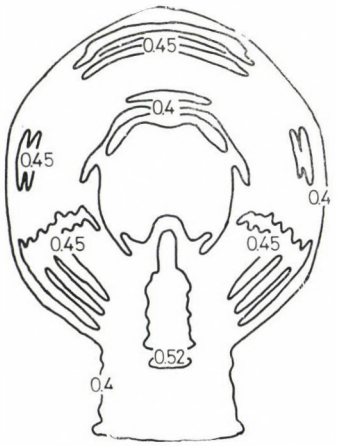

Fig. 1. Electron density map of phage T7. The data give the electron number in 10^{-3} nm^3

sities, one of them is the electron density of the more hydrated, the other is that of the less hydrated nucleic acid (lines denoted with 0.45 and 0.52, respectively).

4. The ordering of the nucleic acid inside the phage head takes place via the interaction with the protein, and this is true not only for the phage but for numerous nucleic acid–protein complexes, i.e. for the nucleic acids in the cell nucleus in the chromosome. According to our experience, if the protein–nucleic acid interaction is disrupted, also this characteristic ordering disappears (Fekete, et al., 1982).

The structural changes in the nucleic acid and nucleoprotein can be followed in various ways. Figure 2 shows the rearrangement process of phage T7 under the effect of temperature increase in the range 37–95 °C. The change was followed in this case by UV absorption. According to the figure, the rearrangement takes place in several stages. The first one is observed at 50 to 60 °C, the other three at 85 to 95 °C. The figure requires two comments: (1) the absorption decrease or increase and the transition temperatures depend strongly on the ion concentration and ionic composition of the solution (Fidy et al., 1983b; Gál, Tóth, 1983); (2) in a homogeneous DNA solution, in the same milieu, the rearrangement takes place in one step at about 88 °C.

Consider now only the first structural transition which is accompanied by hypochromicity. At this temperature the above mentioned fine structure of the small angle X-ray scattering curve gradually disappears, which means that the superhelical and/or liquid crystalline structure disappears under the effect of a high temperature of 50 to 60 °C (Dembo et al., 1982; Fekete et al., 1982).

5. The liquid crystalline ordering is of dominant importance from the point of view of chemical reactions taking place in the system. In the followings we deal shortly with the difference between the photochemical reactions of DNA in isotropic solution studied in the usual way and those of DNA in the phage head. One of the examples to be presented is related to the effect of the far, the other of the near UV range.

Figure 3 shows the kinetics of the UV-photoproduct (structural damage) formation in DNA inside the phage head, and in DNA which had emerged from the phage head. The formation is followed by the loss of the characteristic UV-

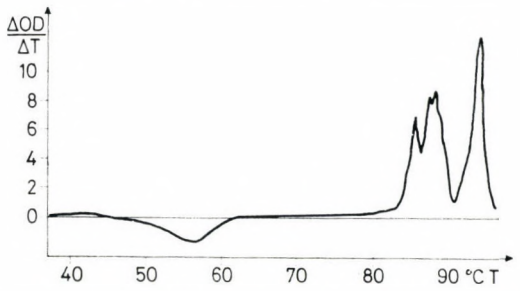

Fig. 2. Variation of the absorption of phage T7 at 260 nm in dependence of temperature (Tris buffer; 0.1 mol/l NaCl)

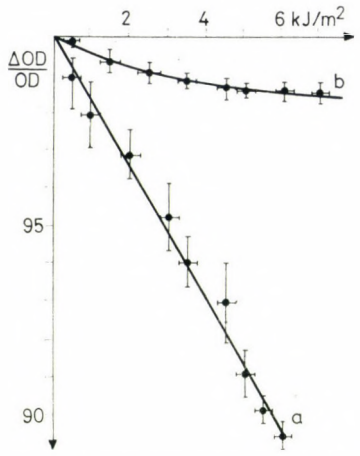

Fig. 3. Damage of T7 DNA under the effect of 254 nm UV light. On the abscissa the incident light energy, on the ordinate the relative change of the optical density measured at 264 nm. Curve *a* relates to the DNA in the phage head, curve *b* to the one emerged from the phage head

absorption (at 260 nm) of intact nucleic acid bases. From the comparison of the curves it can be seen that the nucleic acid ordered inside the phage shows a greater loss of intact bases than the one which had emerged from the head (Fekete, Rontó, 1980).

The other example shows the different behaviour of the nucleic acid in the above mentioned two systems, under the combined effect of physical and chemical agents. We are dealing with the photochemical reaction of trimethylpsoralene used in dermatology and cosmetics and the nucleic acid which is induced by two photons (e.g. at 365 nm), and through the psoralene molecule cross-links are produced in the DNA. The kinetics of the reaction can be followed in Fig. 4. The effectivity of the reaction is characterized by the temperature at which the rearrangement of the DNA double helix takes place. The higher is this temperature, the more stable is the double helix, the higher is the number of cross-links in the DNA. Curve *a* relates to the reaction of DNA within the phage head, curve *b* to that of DNA which had emerged. From the figure it can be seen that the nucleic acid in the phage head is less stable during the reaction, than the other one. In other words: in the first case less, in the latter one more DNA-psoralene cross-links are formed in the given conditions (Fidy et al., 1983a).

In my opinion, the significance of the liquid crystalline state of DNA is emphasized by the practical curiosities related to the presented examples too, especially if the phages are considered as chromosome models. The first UV damage can be induced by the small amount of far UV component of the sunlight reaching the Earth, because the ordered DNA is just the more sensitive. On the other hand the chemical reaction induced by psoralene and near UV light may

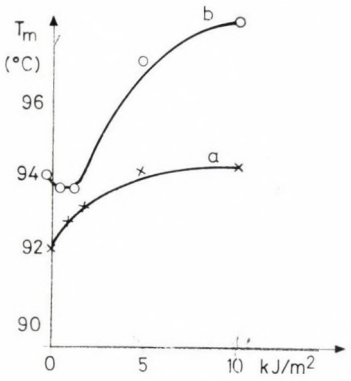

Fig. 4. The damage of T7 DNA induced by the combined effect of 365 nm UV light and trimethylpsoralene. On the abscissa the incident light energy, on the ordinate the temperature of the DNA rearrangement is given. Curve *a* relates to the DNA in the phage head, curve *b* to the one emerged from it

occur as a damage of natural environment, or as a photochemotherapeutical intervention. In this case the therapeutic effect is diminished by the ordered structure.

Finally, one may state again that the chromosome damages may be modelled better by the liquid crystalline nucleic acid than by the one in isotropic solution. In other words: the results obtained on isotropic solution may be applied to the liquid crystalline systems only with restrictions; as the presented examples show, in the presence of the higher order structures one has to take into consideration the modification of the reactions.

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Rotational Dynamics of Proteins in Glycerinated Muscle Fibres[1]

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In the current theories of muscle contraction it is supposed that the contraction is achieved by the sliding motion of filaments of actin and myosin past on each other (Huxley, 1969, 1974, 1980; Huxley, Simmons, 1973). The process is driven by the energy from ATP splitting and is controlled by the presence or absence of calcium ions (Ebashi, Endo, 1968). The cyclic formation and dissociation of cross-bridges requires the large-scale rotational motion of actin and myosin domains, and the interaction affects the conformational state of the protein assemblies. Direct measurements of rotational motion of proteins by motion-sensitive spectroscopic probe molecules provide some insight into the molecular dynamics of proteins during the ATP driven force generating mechanism (Thomas, 1978). Nitroxide spin labels and fluorescence probe molecules tightly bound to proteins have been widely used to study molecular motion in actin, myosin and its fragments, synthetic thick filaments and in supramolecular complexes with actin (Belágyi, 1975, Belágyi et al., 1979; Belágyi, Gróf, 1983; Georgadze et al., 1974; Mendelson et al., 1973; Harvey et al., 1977; Mendelson, 1981; Mendelson, Morales, 1977; Mendelson, Kretzschmar, 1980; Thomas, Cook, 1980). In the present paper we refer mostly to experiments with paramagnetic probe molecules.

One of the key proteins involved in muscle contraction is the myosin localized in the thick filaments. Myosin is a multisubunit protein, each molecule contains two heavy polypeptide chains and four light chains arranged in two globular heads and a long double α-helical rod portion. The latter forms the core of the thick filaments, whereas the globules protrude from the thick filaments and carry the actin-combining sites and contain the ATP-binding domain where the

Abbreviations: DTNB, 5,5'-dithiobis (2-nitrobenzoate); EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; ST EPR, saturation transfer EPR; HMM, heavy meromyosin; NEM, N-ethylmaleimide

[1] Review held at the 12th National Meeting of the Hungarian Biophysical Society (Budapest, 1983).

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Table 1

Rotational correlation time for spin-labelled myosin fragments and glycerol-extracted muscle fibres

Protein moiety	Labelled group	Spin-label	Rotational correlation time in μs
HMM S-1	SH-1	Ṙ-IAA	0.167*
HMM	SH-1	Ṙ-IAA	0.215*
HMM	SH-2	Ṙ-NEM	0.030
muscle fibre	SH-2	Ṙ-NEM	
	H ∥ k		1000**
	H ⊥ k		700

* The value for the rotational correlation time, τ_2, is taken from Thomas and coworkers (1975).

** The different rotational correlation times calculated from the H''/H ST-EPR spectral parameters for the same muscle fibre bundles in two different orientations indicate the anisotropic motion for the attached labels.

hydrolysis of ATP occurs (Goody, Holmes, 1983; Dancker, 1978; Huxley, 1969). It is known that two fast-reacting sulfhydryl groups are located on both globular head regions of the myosin, the use of these sites by substitution with spectroscopic probe molecules has provided valuable information about the muscle proteins in the contraction process (Mendelson, Cheung, 1978; Morales, Botts, 1980; Thomas et al., 1975, 1979, 1980; Mendelson, Wilson, 1982).

The protein molecule in the heavy chain core in the 22 kD segment has two −SH groups, called as SH-1 and SH-2 groups, which are essential for the myosin ATPase activity (Kielley, Bradley, 1956; Sekine, Kielley, 1964; Seidel, Gergely, 1973). Several measurements of the distance between the SH-1 and the SH-2 groups of myosin have been reported indicating that it is about 1.2–1.4 nm (Reissler et al., 1974b). Chemical modification of the most reactive SH-1 group with –SH directed labels such as N-ethylmaleimide or iodoacetamide is accompanied with an increase in the Ca^{2+}-activated ATPase activity with a simultaneous decrease of the K^+ (EDTA)-activated ATPase activity. In the absence of nucleotide the SH-2 groups remain burried and inaccessible to N-ethylmaleimide or other thiol reagents. Subsequent blocking of the SH-2 group leads to a complete loss of the Ca^{2+}-activated ATPase activity. It was demonstrated in previous works that nucleotides (ATP, ADP) accelerate the reaction of the SH-2 groups with N-ethylmaleimide (Reissler et al., 1974a; Seidel, 1972), but they do not protect the SH-1 sites from chemical modification. The enzymatic activities of myosin with labelled SH-2 and regenerated SH-1 groups are similar to those measured if only the SH-1 groups were modified (Seidel, 1969). By varying the conditions it is possible to modify preferentially the SH-1 or SH-2 groups.

In earlier investigations it was found that the mobility of an iodoacetamide spin label bound covalently to the SH-1 thiol group of myosin subfragment-1 (S-1) reflected the motion of the entire protein or a large domain of it (Seidel et al., 1970; Stone, 1973; Thomas et al., 1975). However, during hydrolysis of ATP the local environment of the labelled group exhibited an increase in mobility (Seidel, Gergely, 1971, 1973; Tokiwa, 1971), therefore another labelled group is required in order to monitor the large-scale motion of the protein domains of actin and myosin in the contraction process.

The SH-2 group of myosin labelled with a maleimide label exhibits a remarkably weaker immobilizing effect on the label than the environment of the SH-1 group, but in the supramolecular system of glycerol-extracted muscle fibres a drastically reduced motion of the label at the SH-2 site was observed, which could be due to an induced immobilization of the label within the portion of S-1 containing the label (Table 1, Belágyi et al., 1979). Therefore, it seems that immobilized labels attached to the SH-2 group of HMM in glycerol-extracted muscle may be sensitive probes of molecular motion in the conventional ($10\ s^{-10}$ – $10\ s^{-7}$) and/or saturation transfer EPR time domains ($10^{-7}\ s - 10^{-3}\ s$) in the study of the contraction process.

The rotational correlation time of the maleimide spin label attached covalently to the SH-2 thiol group of HMM was obtained by the extrapolation methods proposed by Shimshick, McConnell (1972) and Mason, Freed (1974). The τ_2 found for the label on isolated HMM was 50 and 35 ns respectively, which shows some residual mobility in the environment of the attached label at the SH-2 thiol site (Figs 1 and 2).

It is important to note that the striated muscle is highly organized structurally, therefore it is expected that the EPR spectrum, which depends on the orientation of the spin labels with respect to the applied magnetic field (H), will reflect the distribution of the oriented spin labels attached to the SH-2 thiol sites.

The first experiments on the orientation dependence of spin labels attached to the proteins in glycerol-extracted muscle fibres were carried out by Cook

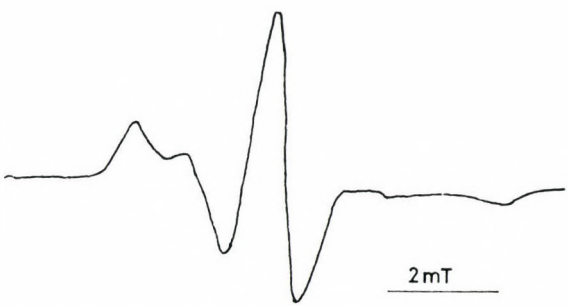

Fig. 1. EPR spectrum of HMM labelled with maleimide spin label at the SH-2 group. The fast-reacting thiol sites (SH-1) were blocked with N-ethylmaleimide as described in the Experimental Procedures. (15 mg/ml HMM, 100 mM KCl, 50 mM Tris-HCl pH 7.5)

and Morales (1969). Later on, we showed using a lower ratio of moles of spin labels to one mole of protein for labelling the myosin SH-1 groups in muscle fibres that the $2p\pi$ orbital of the unpaired electron (z-axis of the magnetic tensors) possesses a perpendicular orientation, rather than a parallel one, to the fibre axis, and the degree of order of the spin labels depends on the mechanical state of the muscle fibres (Fig. 3, Belágyi, 1975).

In order to control whether the spin label is located on the head portion of the myosin molecules we incubated the glycerol-extracted muscle fibres for 24 hours in rigor-inducing solution containing 10 mg/ml spin-labelled HMM. The HMM was labelled with iodoacetamide spin label on the SH-1 thiol sites. After the labelling procedure the fibres were washed in relaxation-inducing solu-

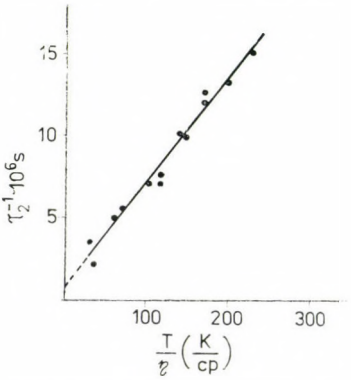

Fig. 2. Plot of the reciprocal correlation time of HMM labelled with maleimide spin labe at the SH-2 thiol site against T/η, where T is absolute temperature and η is the medium vis cosity. The rotational correlation time was calculated according to Freed (1979)

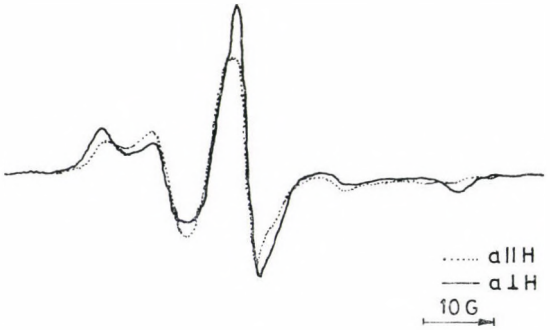

Fig. 3. EPR spectra of glycerol-extracted muscle fibres labelled with maleimide spin label at the SH-1 site of myosin. Spectra were taken in parallel (- - - -) and perpendicular (———) orientation of the fibre axis (a) relative to the applied magnetic field (H). Fibres were kept in rigor-inducing solution during the measurement (100 mM KCl, 1 mM $MgCl_2$, 6.7 mM phosphate buffer, pH 7.0)

tion for 30 minutes and EPR spectra were taken in two orientation. The EPR spectra of fibres loaded with HMM exhibited strong dependence of orientation with respect to the fibre axis. The analysis of the EPR spectra showed that the orientation dependence is similar to that found in glycerol-extracted muscle fibres labelled at the SH-1 thiol site of myosin with maleimide spin label (Belágyi et al., 1978). The same result was obtained by Thomas and Cooke (1980).

It follows from our electron microscopic observation that the HMM subunit of myosin can penetrate the glycerol-extracted muscle fibres, and the interaction of HMM with actin is not restricted to the I-bands, but extends to the overlap regions as well. This finding is in agreement with the earlier observations of Garamvölgyi and coworkers, who decorated actin filaments in myofibrils with S-1 subunit of myosin (Garamvölgyi et al., 1976).

The EPR spectra of glycerol-extracted muscle fibres labelled in the presence of pyrophosphate after DTNB–EDTA treatment with the maleimide spin label at the SH-2 site of myosin (see Experimental Procedures) exhibit strong dependence of orientation with respect to the fibre axis evidencing that the $2p\pi$ molecular orbital of the unpaired electron is oriented almost perpendicular to the long axis of the muscle fibre (Fig. 4).

Labelling the glycerol-extracted muscle fibres with the pyrrolidine maleimide spin labels having increasing chain length between the pyrrolidine ring and the maleimide ring results in a progressive decrease of the orientation dependence

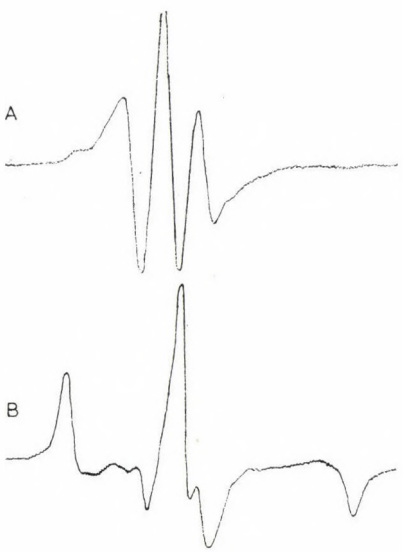

Fig. 4. EPR spectra of glycerol-extracted muscle fibres labelled with maleimide spin label at the SH-2 site of myosin after DTNB–EDTA treatment in two different orientations in rigor state of the muscle fibre. A: fibre axis was oriented parallel to the magnetic field, B: fibre axis was oriented perpendicular to the magnetic field. Sarcomere length of the fibres was (2.3 ± 0.1) μm

(Fig. 5). A strong increase in the mobility of the attached labels was observed using the maleimidoacetamido pyrrolidinyl label. This allows the estimation of the effective depth of the combining site (SH-2 site): it is about 0.9 nm.

It should be noted that the highest degree of order of spin labels in rigor state of the muscle fibres was obtained after DTNB–EDTA treatment, which was regularly used to block the fast-reacting thiol groups including the SH-1 sites before spin-labelling. The reaction of the fibres with N-ethylmaleimide, p-chloromercuribenzoate or dinitrofluoro-benzene before spin labelling with the maleimide derivative in the presence of nucleotides resulted in a significantly lower orientational order of the probe molecules (see Figs 4 and 5).

Fig. 5. EPR spectra of glycerol-extracted muscle fibres labelled with pyrrolidine maleimide spin labels at the SH-2 site of myosin after N-ethylmaleimide treatment in two different orientation of the fibres in rigor state (- - - -, $H \parallel k$; ———, $H \perp k$). a – maleimido-pyrrolidinyloxyl; b – maleimidomethyl-pyrrolidinyloxyl; c – maleimidoacetamido-pyrrolidinyloxyl; d – (maleimidopropil) carbamoyl-pyrrolidinyloxyl

In studying the rotational motion of myosin heads in myofibriles, Thomas and coworkers (1980) used a relatively long (40 hours) treatment of myofibrils with high concentration (50 mM) of $K_3Fe(CN)_6$ after spin-labelling, as suggested by Graceffa and Seidel (1980), in order to have samples in which no or very little amount of weakly immobilized labels were present. Our labelling procedure eliminates the treatment with $K_3Fe(CN)_6$ because the DTNB–EDTA modification leads not only to the blocking of the fast-reacting SH-1 thiol sites but simultaneously reduces the amount of the easily accessible, non specific thiol groups.

The high degree of order shows, that

1. our experimental method allowed the specific labelling of one sulfhydryl group in the protein system of the muscle fibres;

2. in rigor state of the muscle fibres the principal axes of the spin labels are uniformly oriented relative to the fibre axis.

Recently, Cook and Franks (1980) reported that almost all myosin heads are attached to actin in rigor state of the skeletal muscle. The EPR results are consistent with this finding, suggesting a narrow angular distribution of cross-bridges for near uniform angle of attachement to the thin filaments. This conclusion is supported by the experimental observation that the EPR spectra from rigor muscle fibre reflect no motion on the time scale of the ST-EPR technique (Table 1, Gróf, Belágyi, 1984). According to Libertini and Griffith (1970) it is possible to calculate approximately the mean value of the angle between the fibre axis and the principal axis (z) of the spin label: it takes up a 84° angle relative to the fibre axis. Using a gaussian distribution function for label orientation Thomas and Cook (1980) obtained 82° angle for the center of the distribution in the case of maleimide spin label from the simulated EPR spectra.

It is known from the theory of linewidth in magnetic resonance spectra that in strongly immobilized state of the spin labels the outer hyperfine lines of the EPR spectra arise from radicals which are oriented with their magnetic z-axis parallel to the applied magnetic field, whereas the inner hyperfine lines arise from labels with principal z-axis perpendicular to the magnetic field (McConnell, McFarland, 1970; Hubbell, McConnell, 1969). In perpendicular orientation of the labelled muscle fibres with respect to the magnetic field ($H \perp k$), it can be assumed that the number of labels with $z \| H$ is approximately equal with $z \perp H$ by the axial symmetry of the fibres and the attached paramagnetic probe molecules, therefore the spectral feature does not differ significantly from the spectrum of muscle sample, where the fibres were minced to have a random orientation of labels before measurement (isotropic distribution of labels). Supposing a change in the axial order to the cross-bridges (probe molecules) during muscle activity, only a little or no change can be expected in the spectral feature in the perpendicular orientation of the fibre axis relative to the magnetic field, because the spectrum is effectively indistinguishable from the isotropic case. In contrast to that, any change in the motional rate of the label on the time scale of the conventional EPR spectroscopy appears as an increase or decrease in the splitting of the outer extrema ($2A'_{zz}$). However, the parallel orientation of the

fibres ($H \| k$) is optimally sensitive to changes in the distribution of spin labels (or cross-bridges) during muscle activity, because a decrease in the orientational order will be reflected as an increase in the ratio of the two low-fleld peak heights (first vs. second maximum).

The L''/L, C'/C and H''/H parameters defined by Thomas et al. (1976) have routinely been employed to analyse biological systems undergoing very slow isotropic Brownian diffusion (Marsh, 1981; Fajer, Marsh, 1982; Hemminga, 1983). But in the case of partially oriented paramagnetic probe molecules (e.g. oriented fibres, such as the muscle samples) the spectral parameters depend on the orientation of the fibre axis relative to the external magnetic field (Fig. 6). It follows

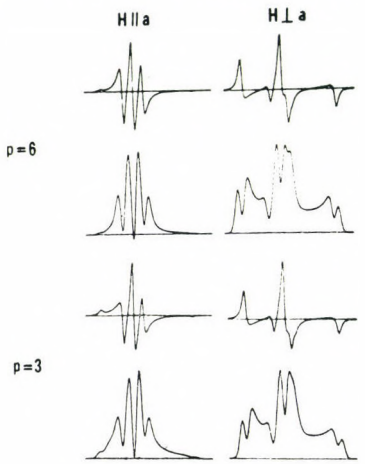

Fig. 6. Simulated conventional and ST EPR spectra of maleimide spin-labelled glycerinated muscle fibre at different degrees of order of spin labels attached to the SH-2 sites of myosin. p is the parameter of the distribution density function, which determines the degree of order of spin labels (Gróf, 1983). At increasing p the partial ordering of the spin labels increases

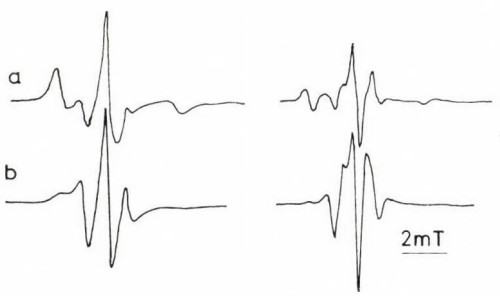

Fig. 7. Conventional (left) and ST EPR (right) spectra of frog nerve in two different orientation of the long axis of the fibres relative to the magnetic field. Fatty acid probe molecules were incorporated into the membrane as described (Gróf, Belágyi, 1983a). a: fibre axis is oriented perpendicular to the magnetic field; b: fibre axis is oriented parallel to the magnetic field

from the results of spectral simulation that significant changes might be expected in the ST EPR spectra even in the absence of any motion, inasmuch as the degree of order of spin labels changes during muscle activity (Gróf, 1983; Gróf, Belágyi, 1984).

In order to have an approximately good estimation of the real rotational correlation time of the attached label in partially oriented systems in the case of very slow molecular motion, we performed experiments on different supramolecular complexes. The systems chosen in our experimental strategy were:

a. nerve fibres, in which fatty acid spin probes are incorporated into the lipid region of the membrane $\tau_2 \sim 10$ ns; Fig. 7; or membrane proteins of nerve labelled with the maleimide spin labels ($\tau_2 \sim 1$ μs; Fig. 8);

b. oriented F-actin filaments, in which the maleimide spin labels are rigidly attached to the cys-373 residue (Elzinga et al., 1973). The effective rotational correlation time is in the submillisecond time range of the molecular motion, $\tau_2 \sim 100$ μs (Fig. 9);

c. muscle fibres, labelled with the maleimide spin labels as described in Experimental Procedures, having rotational correlation times in the millisecond time domain; $\tau_2 \sim 1$ ms (Fig. 10).

From the comparison of the ST EPR spectra of different samples in different orientation (Figs 6 and 11), we may conclude that reliable rotational correlation times can be obtained only in orientation where the z-elements of the magnetic tensors are oriented primarily parallel to the applied magnetic field. We propose, that the rotational correlation time can be estimated in this case according to the known procedure for isotropic distribution of spin labels.

We have shown in these experiments, that in partially oriented systems the degree of ordering of probe molecule can be measured in $H \perp z$ orientation using conventional EPR technique, whereas the $H \| z$ orientation of the samples is very promising for the determination of the rotational correlation time of the labels (protein molecules) in the ST EPR time domain.

In a recent work we have investigated the EPR spectra of the glycerol-extracted muscle fibres labelled with the maleimide spin labels on the SH-2 site of myosin in different mechanical state of the fibres (Gróf, Belágyi, 1984; Gróf et al., 1983). In activating solution, the conventional EPR spectra of the fibres

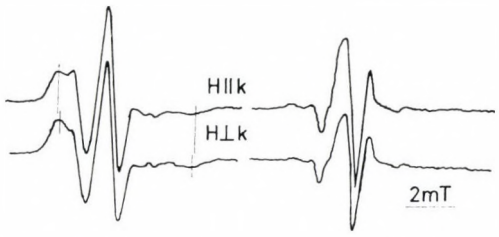

Fig. 8. Conventional (left) and ST EPR (right) spectra of membrane proteins in frog nerve in two different orientation of the long axis of the fibres relative to the magnetic field. The membrane proteins were labelled with the maleimide spin label (Gróf, Belágyi, 1983a)

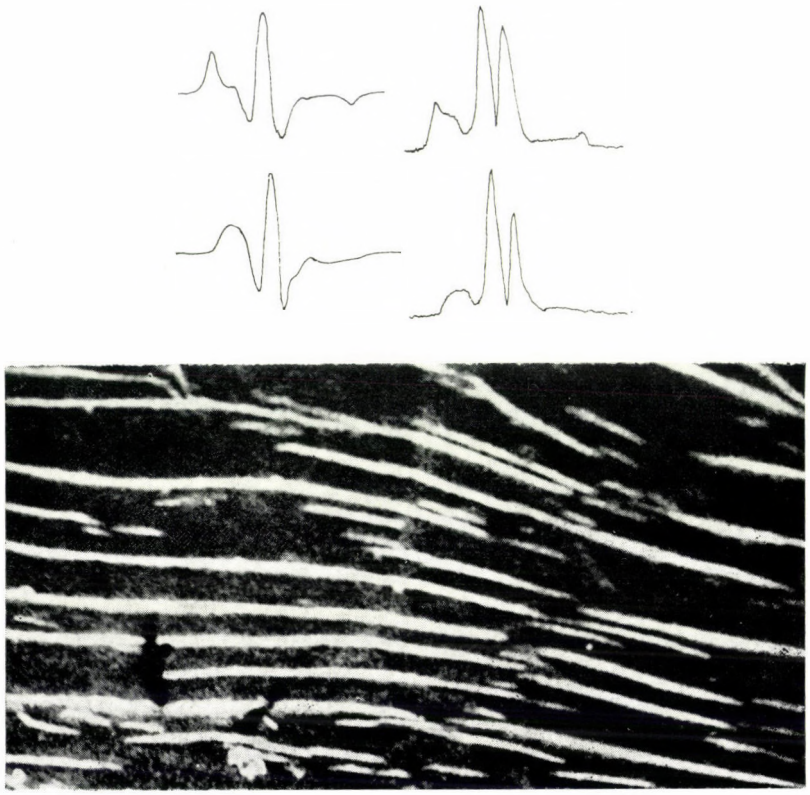

Fig. 9. a) Conventional (left) and ST EPR (right) spectra of oriented F-actin gel in two different orientation of the filament axis with respect to the magnetic field. Actin was labelled at the cys-373 thiol site with the maleimide spin label (Elzinga et al. 1973). Upper spectra: filament axis was oriented parallel to the magnetic field ($H \parallel k$); bottom spectra: filament axis was oriented perpendicular to the magnetic field ($H \perp k$). b) Electron micrograph of oriented associated filaments (×8000)

Fig. 10. Conventional (left) and ST EPR (right) spectra of glycerol-extracted muscle fibres labelled with maleimide spin label at the SH-2 site of myosin

show large changes in the degree of order of spin labels (cross-bridges) toward randomization (Fig. 12). A change in the angle of tilt of spin labels with respect to the fibre axis is probably reflected as a decrease in the order of the spin labels in the conventional EPR spectra. In highly stretched state of the activated muscle

Fig. 11. Conventional (left) and ST EPR (right) spectra of partially oriented biological systems with different rotational correlation times: a) muscle fibres: $H \perp k$, $\tau_2 \sim 1$ ms; b) oriented F-actin gel: $H \parallel k$, $\tau_2 = 100$ μs; c) membrane proteins: $H \perp k$, $\tau_2 = 1$ μs. At the given orientation of the biological system the z principal axis of the magnetic tensors was oriented parallel to the magnetic field (H)

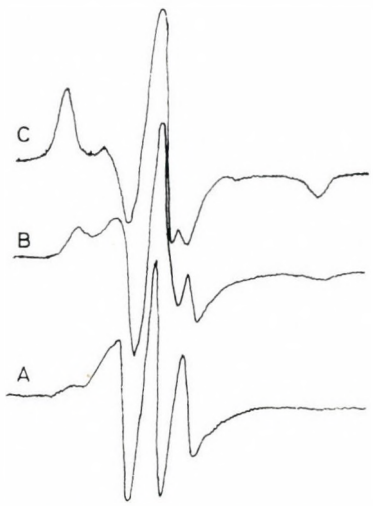

Fig. 12. Conventional EPR spectra of glycerol-extracted muscle fibres labelled with the maleimide spin label after DTNB–EDTA treatment at the SH-2 thiol site of myosin in activation-inducing solution in parallel orientation of the fibre axis relative to the magnetic field: A: rigor state (2.3 ± 0.1) μs/sarcomere; B: contracted state (1.6 ± 0.2) μm/sarcomere; C: minced fibres

Fig. 13. Low-field peaks of the conventional EPR spectra of glycerol-extracted muscle fibres labelled with the maleimide spin label at the SH-2 site of myosin after N-ethylmaleimide pretreatment in parallel orientation of the fibre axis relative to the magnetic field in different state of the fibres; rigor state, (2.3 $\pm$ 0.1) μm/sarcomere (———) activated state (1.6 $\pm$ 0.1) μm/ sarcomere (- · - · -) activated fibres after stretching (3.2 $\pm$ 0.2) μm/sarcomere (- - - -)

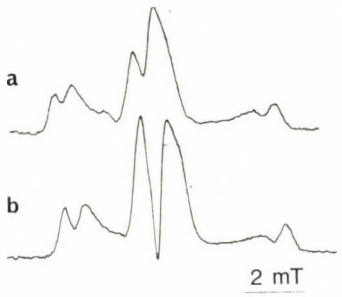

Fig. 14. ST EPR spectra of glycerol-extracted muscle fibres labelled with the maleimide spin label at the SH-2 site of myosin in perpendicular orientation of the fibre axis with respect to the magnetic field. a: rigor state; b: activated state

fibres, when the macroscopic order of the filaments and fibril is much higher than in other state of the fibre bundles, the degree of ordering of probe molecules is smaller than in rigor state of the muscle (Fig. 13). The higher change measured in the degree of order of spin labels in the actively contracted state of the fibres is probably accompanied with a change in the static disorder of the muscle sample.

The large orientation dependence of the EPR spectra observed in rigor state of the fibres is produced by the specific binding of the heads to the thin filaments. Alternatively, the probe orientation is determined by the actual conformation of myosin domain (that holds the label) on actin in the supramolecular complex of the contractile proteins. When the spin-labelled fibres were immersed in activation-inducing solution, and the sarcomere length was allowed to shorten from (2.3 $\pm$ 0.1) μm to (1.6 $\pm$ 0.2) μm or the activated fibres were stretched up to a new length of (3.2 $\pm$ 0.2) μm/sarcomere, we obtained a reduced degree of

order of probe molecules. It was supposed that the resulting spectrum has two components, one representing a population of labels with the same high degree of order of the probe molecules as in rigor state of the fibres, and another corresponding to an isotropic distribution of labels; at molecular level, the former fraction relates to the attached state of myosin heads, whereas the remaining fraction represents the myosin heads in detached state (Thomas, Cooke, 1980; Cooke et al., 1982).

To complete the muscle model it is necessary to check the rate of motion of the protein domains and/or the attached probe molecules. Thomas and coworkers (1980) found on spin-labelled myofibrils that the rotational mobility of heads increased by two order of magnitude in the presence of ATP, therefore they suggested that the source of the disorientation is the rotational mobility of the unattached myosin heads. In the present experiments we observed little sub-millisecond rotational motion in the ST-EPR spectra of oriented fibres in activation-inducing solution ($\tau_2 \sim 400$ μs; Fig. 14). At first, it might appear that these data are in disagreement with the results reported by Thomas and coworkers (1980) and Cook et al. (1982). The major difference between our results and these previous data is that they performed the experiments on myofibrils which shorten free without any load, whereas in our study the muscle fibres exerted tension at a given length. Nevertheless, a simple explanation of our finding might be that the maleimide labels attached to the myosin heads affect somehow the force-generation process, as was found by Mulhern and Eisenberg (1978), and we perhaps observed cross-bridges which had lost their ability to produce tension. However, we showed that the ratio of the ATPase activities of the labelled and control fibres were equal to 1.0 ($\pm$ 0.2) in activation-inducing solution and the tension exerted by the labelled fibres was not smaller than in the case of untreated fibres (Gróf, Belágyi, 1984).

In order to explain the experimental finding, we propose a model which has the essential properties of the cross-bridge model as was first suggested by Huxley (1957) and theoretically formalized by Hill (1974), but the actin has a major role in the cross-bridge cycle. The energy from the ATP hydrolysis, which is localized at the beginning of the reaction in the environment of the ATP binding site, will partially be transferred to a domain of the actin filaments at the further steps of the biochemical kinetic cycle (Fig. 15). New data suggest that ATP hydrolysis appears to occur without dissociation of the actomyosin complex and the myosin head in the refractory state is able to bind weakly to actin (Eisenberg et al., 1980; Brenner et al., 1983). The energy may excite an internal mode of the rotational motion of a domain of the thin filament which, during rotation, causes a constrained disalignement of the myosin heads. The rotation of the myosin heads, which is detected by the ST EPR technique in the sub-millisecond time range, brings about a change in the degree of order of probe molecules in the conventional EPR spectrum. It seems that the disorder of the myosin heads during muscle activity is dynamical on the microsecond or submillisecond time scale. Although our model provides a possible explanation for the EPR data on

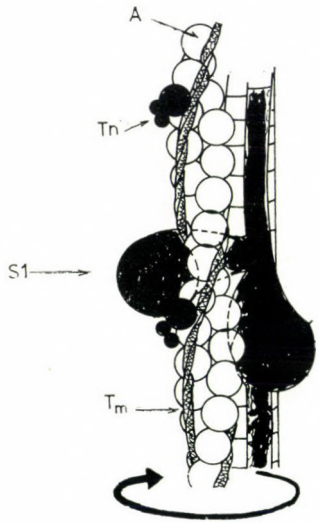

Fig. 15. Schematic sketch of the muscle model. Symbols: *A*: actin, *S* 1: HMM subfragment one, *Tm*: tropomyosin, *Tn*: tropin subunits. Rotation of a domain of the thin filaments generates the elementary motion of the cross-bridges. The motion has an azimuthal and a radial component as well

glycerol-extracted muscle fibres, it is necessary to make further efforts to refine or reject our model for the better understanding of protein dynamics during the muscle contraction process.

Experimental procedures

A. *Preparation and Spin-labelling of Proteins and Muscle Fibre*

Rabbit back and leg muscles were used for protein preparation. Actin was prepared from acetone-dried muscle powder by extraction at 0 °C with 0.2 mM ATP, 0.5 mM dithiothreitol and 5 mM TRIS-HCl buffer, pH 8.5 (Spudich, Watt, 1971). The extraction was followed by two polymerization depolymerization cycles. G-actin was polymerized by the addition of 0.1 M KCl and 0.6 mM $MgCl_2$. Actin was labelled in F-form with N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) maleimide spin label. One mole of spin label reacted with one mole of actin under continuous stirring for 120 minutes over ice. A value of 42.000 daltons was assumed as molecular weight of the monomer actin. Under the experimental conditions used in our study it was shown that cys-373 incorporates more than 90 per cent of the label (Elzinga, Collins, 1977). The unreacted labels were removed by pelleting the actin by ultracentrifugation and thereafter by exhaustive dialysis overnight against the extraction solution without dithiothreitol.

Myosin was extracted from minced rabbit skeletal muscle in 3 vol KCl solution (0.3 M KCl, 0.15 M phosphate buffer, pH 6.5). The crude myosin was purified by alternately lowering and raising the ionic strength of the solution to precipitate and dissolve the protein (Stone, 1970). After the fourth cycle the myosin was clarified by centrifuging at 100 000 × g for 60 minutes at 0°C. HMM was isolated from tryptic digest of myosin (1 : 300 trypsin to myosin weight ratio) according to the method of Lowey and coworkers (1969) and was lyophilized until use.

Before spin-labelling, HMM was allowed to react with NEM or DTNB in order to block the more reactive thiol groups (SH-1). HMM was incubated in a solution containing 100 mM KCl, 50 mM TRIS-HCl (pH 7.5) and 1 mM NEM or DTNB for 120 minutes at 0 °C. The SH-1 blocked HMM was dialysed overnight at 4 °C against the same buffer without NEM or DTNB. For spin-labelling of the SH-2 sites no more than two moles of piperidinyl maleimide spin label per mole of HMM were reacted with the SH-1 blocked HMM in a solution 100 mM KCl, 2.5 mM ADP, 2.5 mM $CaCl_2$ and 50 mM TRIS-HCl (pH 7.5) for two hours over ice. After the labelling procedure the protein was dialysed again as described above.

The glycerol-extracted muscle fibre bundles were prepared from psoas muscle of rabbit according to the method described by Huxley (1963) and Rome (1972) with slight modification (Szöőr et al., 1981). After washing the fibre bundles in 100 mM KCl, 1 mM $MgCl_2$ and 6.7 mM phosphate buffer, pH 7.0 (standard salt or rigor-inducing solution) at room temperature to remove glycerol, the preparations were kept in standard salt solution containing 5 mM DTNB and 5 mM EDTA for two hours at 0 °C to block the more reactive SH-1 thiol groups on myosin. The fibres were washed overnight in the same solution without DTNB and EDTA. The reaction of spin labels with glycerol-extracted muscle fibres were carried out on ice for 120 minutes. For covalent labelling of SH-2 thiol groups on myosin the piperidinyl maleimide or the pyrrolidinyl maleimide spin labels were used in relaxation-inducing medium (100 mM KCl, 5 mM $MgCl_2$, 4 mM EDTA, 10 mM histidine-HCl and 5 mM ATP or 10 mM sodiumpyrophosphate). The piperidinyl maleimide spin label was synthetized and kindly donated by dr. K. Hideg from our laboratory, whereas the pyrrolidinyl maleimide spin labels were purchased from SYVA (USA). The glycerol-extracted muscle fibres were first made to react with 0.5–1.0 moles of spin labels per 10^5 g of protein. The excess unbound labels were removed by washing the fibre bundles in a large amount of standard salt solution for 16 hours at 4 °C. To restore the activity of the SH-1 groups the fibres were kept in rigor-inducing solution containing at least 10 mM 2-mercaptoethanol (more than 100-fold molar excess to the labelled thiol sites) at room temperature.

B. *Miscellaneous*

Protein concentration was determined by measuring the optical extinction with a Hitachi 125 type spectrofotometer.

Viscosity measurements were performed using an Ubbelohde viscometer supplied with a thermostated water-bath at (23.0 ± 0.3) °C. The outflow times for about 1 ml of water was 39.25 s. Five measurements were made on the samples and the average value was used to estimate the relative viscosity.

For electron microscopic observation the specimens were fixed in 2.5 per cent glutaraldehyde, postfixed in 2 per cent osmium tetroxide in sodium cacodylate buffer, pH 7.4, embedded in araldite and stained with uranyl acetate and lead citrate. The sections were made by an LKB ultratome (Ultratom IV, Sweden) and observed in a JEOL 100 C electron microscope (JEOL, Japan).

For measurement of the mechanical properties of the glycerol-extracted muscle fibres a small fibre bundle of about 3–4 mm length and 0.1–0.2 mm in diameter was prepared. The tension exerted by the muscle fibre bundles was measured using a tension transducer mounted on the stative of a micromanipulator (Zeiss, Jena GDR). The sensitivity of the tension transducer is 13 mV/mg and its linearity is better than 1 per cent. The d.c. output was connected to a X-t recorder (ENDIM X-Y-t, GDR).

C. *EPR measurement*

Conventional and saturation transfer EPR spectra were taken with an ERS 220 type spectrometer (Center of Scientific Instruments, GDR). First harmonic in-phase absorption spectra were recorded using 20 mW microwave power and 100 kHz field modulation with amplitude of 0.1 mT for nerve samples, and of 0.3 mT for muscle fibre bundles. Second harmonic out-of-phase absorption spectra were obtained with 85 mW microwave power and 50 kHz modulation frequency of 0.5 mT amplitude, detecting the signal at 100 kHz out-of-phase. The out-of-phase setting was achieved at subsaturating microwave level, usually at about 1 mW, by shifting the phase of the modulation (self-null method, Thomas et al., 1976; Marsh, 1981). The 85 mW microwave power corresponds to an average microwave field amplitude ($< H_1 >$) of 0.025 mT in the central region of the standard tissue cell of Zeiss (GDR). The microwave magnetic field was determined by the perturbing sphere method or with peroxylamine disulphonate using the same sample cell as for the nerve or muscle samples following Fajer and Marsh (1982). Using the center part of the flat cell, small segments of the muscle or nerve fibre bundles (6–7 mm long and 0.2–0.3 mm thick) were packed parallel to each other. Spectra were recorded in two positions, where the long axis of the fibres were oriented parallel and perpendicular to the static magnetic field. The magnetic field calibration was made by nuclear magnetic resonance magnetometer (MJ-110 R Radiopan, Poland). The reproducibility of the measurements for the hyperfine coupling constant was better than 0.02 mT.

A small copper-constantan themocouple was inserted immediately over the sample in the flat cell and during the measurements the temperature was monitored using a digital thermometer. The temperature of the samples was found to be accurate to within ± 1 °C for a given value of temperature.

The EPR spectra of the spin-labelled samples were stored on tape punch. Analysis and simulation of the spectra were carried out with a data processing system based on an EMG 666 type computer (EMG, Hungary) equipped with a visual display system (Tegzes et al., 1978). Spectra were replotted on a NE 2000/666 type plotter (VIDEOTON, Hungary).

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New Trends in Studying Slow Diffusion Processes of the Cytoplasmic Membrane Components[1]

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Its is widely accepted that, in maintaining the cell's operation, the role of the cytoplasmic membrane in the communication between the cell and its environment is not less important than is the barrier function. Here the term, "communication", means both transfer processes required by the metabolism and any event related to the passage of signals from the environment through the membrane (transmembrane signalling).

A great deal of information on the structure of the cytoplasmic membrane is integrated in the fluid mosaic membrane model (Singer, 1974; Singer, Nicolson, 1972). The most basic features of this model can be summarized as follows. The primary structural component of the cytoplasmic membrane is an ordered lipid bilayer with orientation of the phospholipids (coming to an essential portion of the membrane lipids) in such a way that the hydrophobic hydrocarbon chains turn to the inside of the bilayer. Integral and peripheral proteins and glycoproteins associate with the lipid bilayer. These membrane proteins posses different functions, some of them may serve as receptors or antigens, others display a specific enzymatic activity, etc. Under physiological conditions the lipid phase is fluid allowing rotational and lateral movements of the membrane constituent.

Rotation about an axis, parallel to the normal axis of the membrane, as well as lateral diffusion are frequently occurring types of the movement of membrane components while transmembrane mobility is very rare. Both the rotational and lateral mobilities of membrane proteins are greatly hindered by various interactions or combinations of them making these movements relatively slow compared to similar motions in low viscosity aqueous phase (Nicolson, 1976). The high viscosity of the lipid bilayer is one of the major reasons slowing down the rate of the protein diffusion in membranes. On the other hand, the very high protein concentration within the cellular membrane (Bretcher, 1971; Morrison

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et al., 1974) often facilitates the formation of protein-protein association[1]. In certain cases large size filamentous meshwork can be formed on the cytoplasmic surface of the membrane which may also slow down the lateral diffusion of the transmembrane proteins (Steck, 1974; Nicolson, 1976). An additional restraint is connected with the cytoplasmic control of the cell surface. Elements of the cytoskeletal system may interact with membrane proteins and this "anchoring" frequently gives a contribution to the complex restraining mechanisms preventing some intramembraneous proteins from free movement.

It is known that cells respond very specifically to numerous extracellular signals. A complex cellular response may contain many elementary steps. As a result of such signals (e.g., binding of appropriate ligands to their receptors) the permeability of the cytoplasmic membrane may change resulting in an altered transport of ions and/or metabolites through the membrane. These events may cause a shift in the steady state intracellular concentration of certain ions which can activate enzymes or enzyme systems leading to an increased rate of protein and/or nucleic acid synthesis.

The elucidation of the structure and function or the detailed mechanism of a complex response of a cell to specific triggering signals is a widely studied problem in the molecular and cell biology. Lateral and rotational motion of membrane components are often of central importance in allowing certain interactions between different constituents of the cytoplasmic membrane and/or other cell components. On the other hand, changes in the interactions during a specific response of the cell may reflect in altered rotational or lateral mobility of selected membrane proteins.

These facts underline the importance of the investigations of the dynamic features of membrane components in the elucidation of the mechanism of a complex cellular response.

This review will summarize some of the luminescence spectroscopic methods enabling us to study the proximity relationships (complex formation) as well as the lateral and rotational mobilities of membrane proteins.

Determination of intermolecular distances using fluorescence resonance energy transfer measurements

Information on the aggregation state of the proteins, glyco- and lipoproteins of the cytoplasm can be gained, among others, with methods enabling us to measure average separation distances between the appropriate membrane components. According to the theory of the dipole-dipole energy transfer in the case of a suitable donor-acceptor pair the transfer efficiency E, is a function of spectral

[1] The antibody blocking assay by Boyse et al. (1968) was one of the first methods yielding experimental evidence for the existence of close physical association between membrane proteins. Flaherty and Zimmerman (1979) further developed this method and, on the basis of the results, they suggest a biological significance of the "receptor pattern" — a consequence of protein-protein association.

parameters and the donor-acceptor separation

$$E = R_0^6/(R^6 + R_0^6) \tag{1}$$

where R_0 means the characteristic separation distance at which energy transfer efficiency between the two fluorophors is 50 per cent. The numerical value of R_0 can be calculated from spectral and structural data according to (e.g., Cantor and Schimmel, 1980):

$$R_0^6 = 8.8 \cdot 10^{-25} n^{-4} \kappa^2 \phi_D J \tag{2}$$

where n is the refractive index of the medium in which the energy transfer takes place, κ^2 is the orientation factor for the transition dipole moments of the donor and acceptor, ϕ_D is the quantum yield of the donor in the absence of acceptor, J is the spectral overlap integral.

There exist several ways for the experimental determination of the transfer efficiency. The numerical value of E can be calculated:

a) from the decrease of the life time of the donor in the presence of the acceptor;

b) from the decrease of the intensity of the emission of the donor because of the energy transfer;

c) from the sensitized fluorescence of the acceptor (i.e. from the increased acceptor fluorescence due to the energy transfer, excited in the donor absorption band).

The previous methods are routinely used for the determination of intramolecular and intermolecular distances between specific sites in molecular systems (Stryer, 1978). Each of these methods requires the measurement of fluorescent parameter(s) of a double (with both donor and acceptor) labeled and a single (either with donor or acceptor alone) labeled sample.

Any of the enlisted means for determination of E is principally applicable for average distance measurements on cell surfaces. The specificity of the donor and acceptor labeling in cellular system can be assured by the incubation of the cells with donor and/or acceptor conjugated ligands. These conjugated ligands bind to the receptors (or specific binding sites) thus allowing to carry out distance measurements (Fernandez, Berlin, 1976; Dale et al., 1981). Numerous publications report on E measurements of that type. These measurements, however, yield transfer efficiencies averaged over the entire cell populations. It seems quite reasonable that one is anxious to carry out similar measurements on single cells in order to be able to detect differences between subpopulations of a heterogeneous sample. The heterogeneity can be connected either with the very kind of the cells or their functional states. For this purpose we have developed a flow cytometric method for the energy transfer measurement (FCET) being capable of determining the absolute transfer efficiency on single cells.

The determination of the numerical value of E on a single, double labeled cell generally demands several measurements. First of all, the emission intensity

of the acceptor has to be measured with excitation in the donor absorption band. In our experiment fluorescein (fluorescein isothiocyanate – FITC – conjugated ligands) served as donor and rhodamine (tetramethylrhodamine-isothiocyanate – TRITC conjugated ligands) as acceptor. With this transfer pair, however, (and very often also with other transfer pairs appropriate to measure energy transfer) only a fraction of the detected intensity is related to energy transfer processes because rhodamine emission can be excited in the donor absorption band, too. In order to correct for the directly excited acceptor fluorescence a second intensity determination has to be completed. It is advisable to carry out this second fluorescence intensity measurement on the double labeled sample with excitation at a wavelength where the donor displays a negligible absorbance. This second intensity reading and the measured excitation spectrum of the acceptor fluorescence enable us to perform the mentioned correction. To express the probability of the energy transfer in percents the corrected transfer signal has to be normalized also to the number of the donor excitation events. To do this the donor emission (with excitation in the donor absorption band and detection in its emission band) has also to be measured.

Because of the reasons outlined above in our experiments carried out on a fluorescence flow cytometer we detected three fluorescence intensities from each single cell. We focused two light beams of an argon ion laser (with wavelengths of 488 nm and 514 nm, respectively) on the sample stream carrying the cells in such a way that the cells intersected the two focused beams with a time difference of 30 μs. Thus, the excitations of the same cell at these wavelengths were separated in time. This fact and the detection of the emission in the donor and acceptor emission band separately (using on appropriate optical system) made it possible to measure three independent fluorescence intensities from each analyzed cell. From these I_1, I_2 and I_3 intensities the efficiency of the energy transfer can be calculated as follows:

$$\frac{E}{1-E} = \left\{ \frac{I_2 - S_3 I_3}{\left(1 - \frac{S_3}{S_1} S_2\right) I_1} - S_1 \right\} \frac{1}{\alpha} = A \tag{3}$$

$$E = \frac{A}{1+A} \tag{4}$$

where α, S_1, S_2 and S_3 are parameters characterizing the donor-acceptor pair and the detection system. These parameters do not depend on the transfer efficiency, their numerical values can be measured by analyzing cells labeled with donor alone and acceptor alone, as it is described in more details elsewhere (Trón et al., 1984).

FCET has numerous advantages over the steady-state fluorescence energy transfer measurements in cell suspensions. FCET is capable to detect inhomogeneity in an analyzed cell population allowing single cell transfer efficiency

determinations. The results of FCET measurements are not affected by the presence of unbound "free", donor and acceptor conjugated ligands, as in a flow cytometer the illuminated "sensitive" volume is of the order of 10–100 pl and even from this fairly small volume a substantial part is given by the nonfluorescent sheeth fluid.

Thus, using FCET, it is possible to analyze the cells, without any washing, immediately after the incubation with the labeled ligands is completed. This advantageous feature is of great importance in cases when the ligand off rate is high and any working step in the protocol would significantly perturb the binding equilibrium. Simultaneously with the measurement of the three fluorescence intensities required for an E determination FCET makes it possible to detect additional parameters, too. As a result of such combined measurements the correlation of energy transfer efficiency with other cellular parameters can be investigated. The accuracy of the E data is not influenced by cell debris since data collection can be triggered by light scattering signal (indicating the size of the analyzed objects) above a set threshold.

Measurement of the lateral diffusion of membrane components

Frye and Edidin (1970) were the first to observe the lateral movement of intramembrane proteins. They have found that after cellular fusion the surface markers of the two cells were redistributed on the whole surface of the heterokaryon. Peters et al. (1974) documented that the emission of fluorescein conjugated ligands bound to the cytoplasmic membrane can irreversibly be quenched ("bleached") exposing the cells to a brief, very intensive light pulse. Having focused this bleaching beam to a small ($\phi \cong 1$–2 μm) diameter portion of the cell surface the photobleaching was limited to that very spot. The emission detected from the same area increased in time as nonbleached fluorophors diffuse into the monitored area from the part of the membrane outside the primary bleached region. The technique is called FRAP (fluorescence recovery after photobleaching). The analysis of the time course of the fluorescence yields the lateral diffusion constant. The method itself and the results with FRAP have already been reviewed several times (Webb, 1978; Cherry, 1979; Jacobson, 1980; Edidin, 1981; Jacobson, Wojcieszyn, 1981; Peters, 1981; Schlessinger, Elson, 1981; Webb et al., 1982; Axelrod, 1983).

The FRAP technique has further been developed and modified several times. In some cases the firm localization is not satisfactory, the position of the cells may change during the experiment. This difficulty can be overcome by reflecting the monitor beam into the microscope with the help of a rotating mirror. The periodical motion of this mirror keeps the scanning beam moving along the cell surface. The minimum of the detected fluorescence signal corresponds to the coincidence of the beam with the bleached area. The successive intensity distributions stored in the memory of a computer are analyzed fitting their minima to one another. The technique has been modified in order to make it capable to distin-

guish between random diffusion and systematic flow processes. According to this modified version a "pattern photobleaching" is made producing bleaching at different places and measuring the recovery kinetics from all of these bleached spots. The pattern type bleaching and recovery measurements are realized using a computer controlled moving mirror.

Several methods have so far been developed to analyze the FRAP results (Axelrod et al., 1976; Koppel, 1979, 1980).

Measurement of the rotational mobility of membrane proteins

A common feature of all methods capable of measuring rotational diffusion constants of macromolecules is the selection of an ordered subpopulation from a randomly aligned ensemble. The aligned subpopulation becomes gradually randomized due to the rotational Brown motion in systems with rotational freedom of movement. This process manifests in the time dependence of properly chosen parameters. In the techniques discussed below the selection of the mentioned ordered subpopulation is accomplished by the so called photoselection. The ordered character of the photoselected subpopulation follows from the anisotropic nature of the absorption as the probability of the selection by a plane polarized excitation beam increases with decreasing the angle between the absorption dipole of the molecule and the electric vector of the incident light. The ordered nature of an ensemble of molecules of luminescent character is reflected in anisotropic emission or dichroic absorption. On the basis of this the rotational mobility of macromolecules can be suitably determined by measuring the time dependence of the emission or the absorption of the sample after the photoselection. The emission and absorption anisotropy is defined as

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)} \tag{5}$$

$$r(t) = \frac{A_{\parallel}(t) - A_{\perp}(t)}{A_{\parallel}(t) + 2A_{\perp}(t)} \tag{6}$$

where $t = 0$ at the end of the photoselection, the $I_{\parallel}(t)$ and $I_{\perp}(t)$ are the fluorescence intensities at time t, detected through a polarizer oriented parallel or perpendicular, respectively, to the polarization of the excitation light. Similarly $A_{\parallel}(t)$ and $A_{\perp}(t)$ are the absorbances at time t for light polarized parallel and perpendicular with respect to the polarization of the photoselecting excitation light.

As a result of the photoselection the selected population gets into a spectroscopic state the absorption or emission parameters of which differ from those of the nonselected part (residing usually in the ground state) of the whole population. The time range, within which the randomizing rotational Brown motion can be investigated, is limited by the life time of this spectroscopic state, as any result of the photoselection disappears with the depletion of this state. This fact explains

why the measurement of the fluorescence anisotropy decay, although frequently used for the measurement of rotational diffusion constants of macromolecule in aqueous solutions, does not yield information on the rotational Brown motion of membrane proteins. Rotational correlation times of these proteins fall usually into the μs–ms time interval and the processes occurring in this time range do not cause any decrease of the anisotropy during nanoseconds, the life time of the S_1 state involved in fluorescence. Investigation of such slow rotational motions can be carried out by making use of a spectroscopic state with life time of the order of μs–ms, or longer.

Consequently, it seems plausible to measure the slow rotational motion of proteins embedded into membranes of high viscosity with depolarization techniques, in the case of which the photoselected oriented subpopulation is generated in the triplet state having ms–s life time. There are a few methods developed for this purpose.

Decay of phosphorescence anisotropy

The measurement of time dependent anisotropy of phosphorescence can be regarded as an analogue of the fluorescence anisotropy decay technique. Comparing the two methods, however, quite a number of differences of various origin can be noticed. One of the most essential dissimilarities is that fluorescence can usually be excited with much higher efficiency than phosphorescence. This is why phosphorescence anisotropy decay can not be measured using a method analogous to the nanosecond single photon technique. More precisely it is connected, besides the above mentioned reason, also with the fact, that the time between repetitive, pulse-like excitations should be enough for the complete depletion of the triplet state. In the case of a triplet life time of several ms this would set the upper limit of the repetition frequency below 100 Hz which in turn would dramatically increase the time of data collection.

Another difference between the two methods can also be related to the long life time of the triplet state. Samples used for phosphorescence measurements have to be carefully deoxygenized otherwise the oxygen, a potent triplet quencher, would very much shorten the life time of the photoselected oriented subpopulation. Thorough deoxygenization is an absolute requirement when using any technique based on the long life time of the triplet state.

Triplet probes applied for phosphorescence depolarization measurements should have intersystem crossing transitions of high probability. Quoting examples we recall the triplet yield of some halogenized fluorescein derivatives: 0.46 for dibromo fluorescein: 0.71 for tetrabromo fluorescein (eozin; Bovers, Porter, 1967); 1.0 for tetraiodo fluorescein (erythrosin; Johnson, Garland, 1982). Besides the high triplet yield, good phosphorescent triplet probes show high phosphorescence quantum efficiency, too. Phosphorescence anisotropy decay has been applied for studying the rotational mobility of the epidermal growth factor receptor (Zidovetzki et al., 1981), acethyl choline receptor (Bartholdi et al., 1981) and H-2K antigen (Damjanovich et al., 1983).

The essential features of the equipment used in measurements of this type have already been described (Austin et al., 1979; Jovin et al., 1981; Zidovetzki et al., 1981). The instrument consists of, among others, a transient recorder controlled dye laser as an excitation light source. Recorded spectra are stored in the memory of the transient recorder. The measuring system has the capability of blocking the photodetector during the excitation pulse, in order to avoid photomultiplier damage because of the very high level of the prompt fluorescence. The phosphorescent intensities $I_{\parallel}(t)$ and $I_{\perp}(t)$ are measured alternately in many cycles in order to minimize distortion resulting from instrumental instability ("drift") on the calculated time dependence of the anisotropy. It is necessary to repeat $I_{\parallel}$ and $I_{\perp}$ measuring cycles to reduce the noise of the recorded spectra, otherwise the analysis would yield parameter values with great standard deviations. The analysis of $r(t)$ computed from the stored $I_{\parallel}(t)$ and $I_{\perp}(t)$ functions usually means the fitting of experimental data to a multiexponential decay of the form:

$$r(t) = r_{\infty} + (r_0 - r_{\infty}) \cdot \Sigma\, a_i\, e^{-t/\Theta_i} \tag{7}$$

where r_0 is the initial value of the anisotropy (at $t = 0$), r_{∞} is the limiting anisotropy measured at time $t \gg \Theta_i$, Θ_i denote the rotational correlation times with relative amplitudes of a_i. The rotational diffusion constants may be evaluated from the Θ_i values according to Tao (1969).

Decay of the anisotropy of delayed fluorescence

There are triplet probes with very small energy differences between their S_1 and T_1 states so that the $T_1 \rightarrow S_1$ transitions are thermally activable. Using such probes the detection of the populated triplet state is also possible by the measurement of the delayed fluorescence accompanying the $T_1 \rightarrow S_1$ transitions. The instrumentation needed is practically the same as the one used for phosphorescence measurements (Jovin et al., 1981; Garland, Moore, 1979). This technique has mainly been applied with eosin triplet probes.

Decay of the transient dichroism

The order of the photoselected triplet population can also be measured by determining the dichroic absorbance (due to triplet-triplet transitions) of the same subpopulation. In transient dichroism measurements triplet states are detected from absorbance changes in the sample following the photoselection. At appropriate wavelengths an increase in absorbance occurs owing to a transition from the T_1 state to a triplet state of higher energy. As long as the aligned photoselected subpopulation is not randomized by the rotational Brownian motion, at time t after the excitation flash, $A_{\parallel}(t)$, the transient absorbance changes for light polarized parallely with respect to the polarization of the excitation, is usually bigger than $A_{\perp}(t)$ (that for light polarized perpendicularly with respect

to the former direction). The system of the excited states of the related chromophors usually ensures the measurement of the singlet-singlet and triplet-triplet transitions at different wavelengths separately. The method has been developed by Cherry and his coworkers and has been used for the investigations of numerous biological systems (Cherry, 1978, 1979). Using this method the rotational correlation times can be evaluated by fitting the experimental $r(t)$ curve generated according to equation (6) to a theoretical function (eq. 7). The rotational diffusion constants may be calculated as it was mentioned in connection with the phosphorescence depolarization method. The sensitivity of the transient dichroism measurements is lower than that of the methods using light emission since in the former case normally small changes are measured on a relatively high background.

The experimental equipment required for this type of measurements has been described in details by Cherry (1978).

Anisotropy decay of ground state depletion

The method developed by Garland and his group (Johnson, Garland, 1981, 1982) outstands with its highest sensitivity in the detection of slow rotational Brownian motion in comparison with other techniques suitable for this purpose. The high sensitivity is a common consequence of the facts that the monitorization of the photoselected aligned subpopulation is realized by measuring emission rather than absorption and, in addition, instead of measuring phosphorescence the anisotropy of the fluorescence (the absolute quantum efficiency of which can be close to 1) is detected.

In all of the depolarization methods involving triplet states the brief excitation with a polarized beam results in a triplet population with non-random distribution of molecular orientation. As this photoselected aligned subpopulation is missing from the originally random entire population the very fraction of the original ensemble left in the ground state also displays a certain kind of non-random orientation distribution. This "inversely" oriented fraction of the luminophors can also be characterized with an anisotropy whose decay yields information with respect to the time kinetics of the randomizing rotational Brownian motion. In the case of the ground state depletion method the anisotropy is monitorized by measuring the anisotropy of the fluorescence as it was mentioned above. The method necessitates that, after the polarized "depletion light pulse", a remarkable portion of the total amount of luminophors (at least 20 per cent, or so) be in the triplet state. The depletion of the ground state has to occur in a short time relative to the time scale of the correlation times of the rotational process to be studied. This condition requires depletion light pulses of very high energy density which, even if using laser light sources, can be achieved only by light-focusing. This demand nearly offers the application of microscopic systems for this purpose. Microscopy, however, implies certain of disadvantages because the achievement of acceptable statistics requires lengthy experiments. On the other hand, it has

an advantage, i.e. it enables us to investigate individual cells, resulting in well defined and guaranteed homogeneous samples.

A triplet probe appropriate for the ground state depletion measurement has to meet two requirements. A 20 per cent depletion demands a high triplet yield, ϕ_t, the high sensitivity monitorization of the ground state subpopulation requires, at the same time, a high fluorescence quantum yield, ϕ_f. However, ϕ_t and ϕ_f are interdependent, the increase of the numerical value of either of them is possible only at the expense of the other. The compromise can not be avoided since the energy available for the photoselection is limited by the power of the laser and the values of the rotational correlation times of the entities to be studied and, conversely, the intensity of the monitor beam can only be rised till it does not cause any substantial ground state depletion. Principally (based on practical considerations), detection of the triplet state by fluorescence depletion becomes more sensitive as the ratio of ϕ_f/ϕ_t increases. This is so, because the measuring beam intensity can be increased without causing significant further ground state depletion if ϕ_t is low enough. In the applications completed so far the highest sensitivity of the method was obtained with eosin, although the more favourable ϕ_f/ϕ_t ratio of the rhodamine derivatives gives rise to a suspicion that they can surpass eosin derivatives in maintaining higher sensitivity. It should be noted at the same time, however, that at high ϕ_f/ϕ_t a ground state depletion of a given extent (e.g. 20–30 per cent) may necessitate very high energies.

In the previous paragraphs optical techniques have been reviewed by which dynamic features of membrane components can be investigated. We do believe that future experiments to be carried out using these techniques will give a substantial contribution to a better understanding of the membrane phenomena especially of the relationship between structural and functional properties of cellular membranes.

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Delayed Fluorescence and Phosphorescence of Flavomononucleotide Stabilized by Poly(vinyl alcohol) Matrix

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Phosphorescence spectra at 103 K and delayed fluorescence and phosphorescence spectra at 296 K of flavomononucleotide (FMN) stabilized by poly(vinyl alcohol) PVA matrix have been measured. At 103 K the FMN monomer and excimer exhibit phosphorescence bands at about 620 and 640 nm, respectively. At 295 K the delayed fluorescence and phosphorescence of the FMN excimer exist together, revealing that FMN in the excited triplet state is an energetically well-isolated molecular complex which may play an important role in photobiological reactions.

Introduction

Flavin molecules can form molecular complexes (dimers or higher multimers) in ground electronic states. Under specific conditions the interaction between flavin molecules in the ground state and flavin molecules in the excited state can lead to excimer formation. The mechanism of complex formation has not been clearly explained as yet (Drabent, Grajek, 1983; Jelinek, 1982; Jung-Do Choi, McCornick, 1980; Wehry, 1976; Foster, 1979; Nishimoto et al., 1980; Shulga, 1982; Slifkin, 1971; Walsh et al., 1980). It was found that FMN molecules in PVA film form excimers which emit fluorescence at room temperature. The fluorescence maxima were at longer waves for excimers than for monomers (Drabent, Szalay, 1984; Drabent, Laczkó, 1984).

The aim of this paper was the investigation of the excimer-delayed fluorescence and phosphorescence at low temperature and at room temperature.

Experimental materials

FMN ($C_{17}H_{20}NaO_9P$) from Fluka AG and coarse-grained PVA powder from Loba-Chemie, Vien-Fischamend, were used. FMN was dissolved in an aqueous solution of PVA and isotropic films were obtained by water evaporation

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(Drabent, Olszewska, 1981; Drabent, Szalay, 1984). The concentrations of FMN in PVA matrices were: $8 \cdot 10^{-5}$, $4 \cdot 10^{-4}$, or $4 \cdot 10^{-3}$ FMN per PVA (g/g).

Methods

Delayed fluorescence and phosphorescence at room temperature and at low temperature were measured according to our previous methods, as outlined in Fig. 1 (Mieloszyk, Siodmiak, 1983). The monochromatic exciting light produced by a Xe-lamp is modulated by a chopper and a monochromator and illuminates the sample. The emitted light passing through the monochromator is measured by a photomultiplier and a recorder or an oscilloscope is used for measuring the decay curves. The K201 type recorder can be connected with the DMR4 type excitation monochromator to measure the excitation spectra. The polarized emission spectra and polarized excitation spectra can be determined by using the polarizers P_1 and P_2. A thermopile with a galvanometer measures the temperature of the sample.

Phosphorescence spectra Ph (λ_{em}), polarized phosphorescence spectra r_{Ph} (λ_{em}) and polarized excitation spectra r_{Ph} (λ_{exc}) were determined at 103 K, while delayed emission spectra F (λ_{em}), polarized delayed emission spectra r (λ_{em}) and polarized excitation spectra r (λ_{exc}) were measured at 295 K.

The delayed emission spectra were corrected for reabsorption. The experiments were performed with $\lambda_{exc1} = 440$ (near the absorption maximum) and $\lambda_{exc2} = 490$ nm (long-wave absorption region, see Fig. 3).

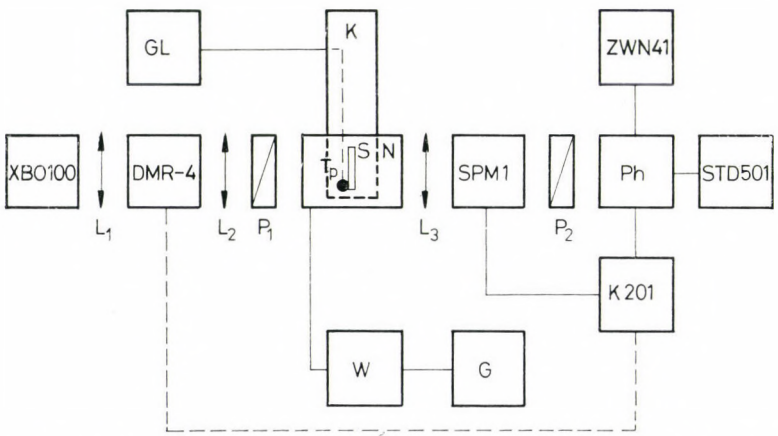

Fig. 1. Outline of the apparatus for measurement of the phosphorescence and delayed fluorescence spectra. XBO 100 — xenon lamp XBO, 100 W; L_1, L_2, L_3 — lenses; P_1, P_2 — polarizers; DMR-4 — excitation monochromator; K — cryostat; S — sample; H — chopper; W — power amplifier; G — audio-frequency generator; T_p — thermopile; GL — galvanometer; SPM-1 — monochromator; P_h — photomultiplier (Centronic Q 4283BA); ZWN41 — high-voltage power supply; K201 — recorder; STD-501 — oscilloscope

Results and discussion

1. Phosphorescence spectra at 103 K.

A previous study has indicated that, at least for the concentrations used in this study, FMN monomers and excimers exist together. The fluorescence band of the excimer is at longer waves than that of the monomer (Jelinek, 1982). The phosphorescence bands show similar behaviour, especially for λ_{exc2} (Fig. 2). This indicates that the phosphorescence at longer waves (about 640 nm) is mainly emitted by excimers of FMN stabilized by the PVA matrix, while the short-wave phosphorescence (about 620 nm) is mainly due to FMN monomers. For all FMN concentrations used, the phosphorescence band excited by λ_{exc2} was situated at longer waves than that for λ_{exc1}. This may be due to a higher efficiency of FMN excimer emission for $\lambda_{exc2} = 490$ than that for $\lambda_{exc1} = 440$ nm.

The phosphorescence anisotropies are near zero (within an error of ± 0.02) in the whole emission band and for all concentrations and therefore we do not give graphs. This result suggests that the phosphorescence transition moment of the FMN excimer is oriented similarly to that for the FMN monomer.

The previous conclusions are confirmed by the polarized phosphorescence excitation spectra (Fig. 3). These are given for the concentrations $8 \cdot 10^{-5}$ and

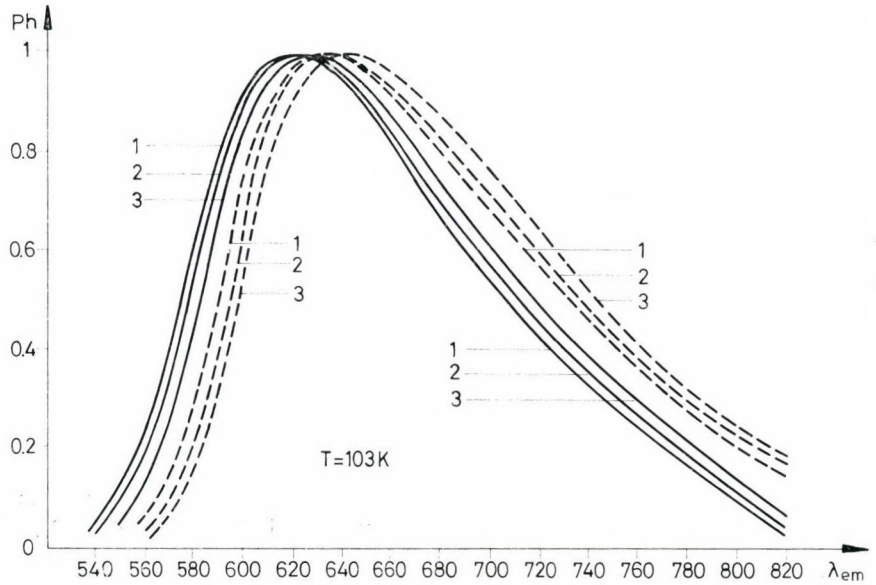

Fig. 2. Phosphorescence of FMN in PVA matrix at 103 K for different FMN concentrations and exciting wavelengths. Phosphorescence spectra Ph (λ_{em}); concentrations: $8 \cdot 10^{-5}$ g/g (1), $4 \cdot 10^{-4}$ g/g (2), $4 \cdot 10^{-3}$ g/g (3); exciting wavelengths: 440 nm (solid line), 490 nm (dashed line)

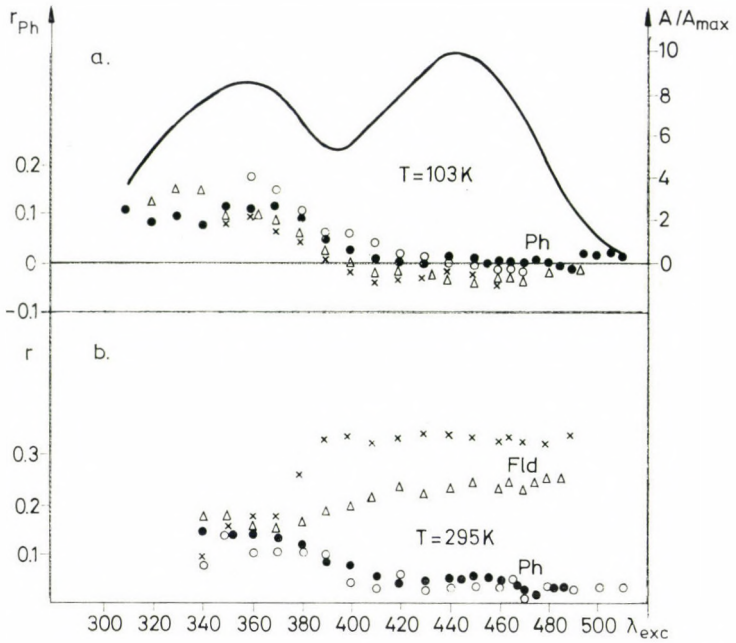

Fig. 3. Polarized phosphorescence excitation spectra of FMN in PVA matrix for different FMN concentrations and observation wavelengths (λ_{em}); a) r_{Ph} (λ_{exc}) at 103 K, concentrations: $8 \cdot 10^{-5}$ g/g, observation 570 (×××) and 700 nm (○○○); $4 \cdot 10^{-3}$ g/g, observation 570 (△△△) and 700 nm (●●●); relative absorption spectrum of FMN in PVA matrix A/A_{max} (solid line); b) r(λ_{exc}) 295 K, observation 495 nm, concentrations $8 \cdot 10^{-5}$ g/g (×××), $4 \cdot 10^{-3}$ g/g (△△△); observation 700 nm, concentrations $8 \cdot 10^{-5}$ g/g (○○○), $4 \cdot 10^{-3}$ g/g (●●●), pH = phosphorescence, Fld = delayed luminescence

$4 \cdot 10^{-3}$ g/g. For comparison the relative absorption spectrum of FMN in PVA film (with band maxima at 445 and 362 nm) is also shown. As is seen in Fig. 3, the emission was excited from the region of the first absorption band.

2. Phosphorescence and delayed fluorescence spectra at 295 K.

The delayed fluorescence and phosphorescence appear at 295 K and their bands shift toward longer waves with increasing dye concentrations (Fig. 4a). The long-wave delayed fluorescence band corresponds to that of prompt fluorescence of FMN excimers measured previously at 295 K (Jelinek, 1982). The relative phosphorescence intensities of the monomer and the excimer (i.e. for low and high concentrations) are comparable to their delayed fluorescence intensities. The relatively high intensity of the phosphorescence at room temperature suggests that in the excited triplet state the FMN excimer stabilized by PVA can be treated as an energetically well-isolated molecular system. The energetically stable FMN

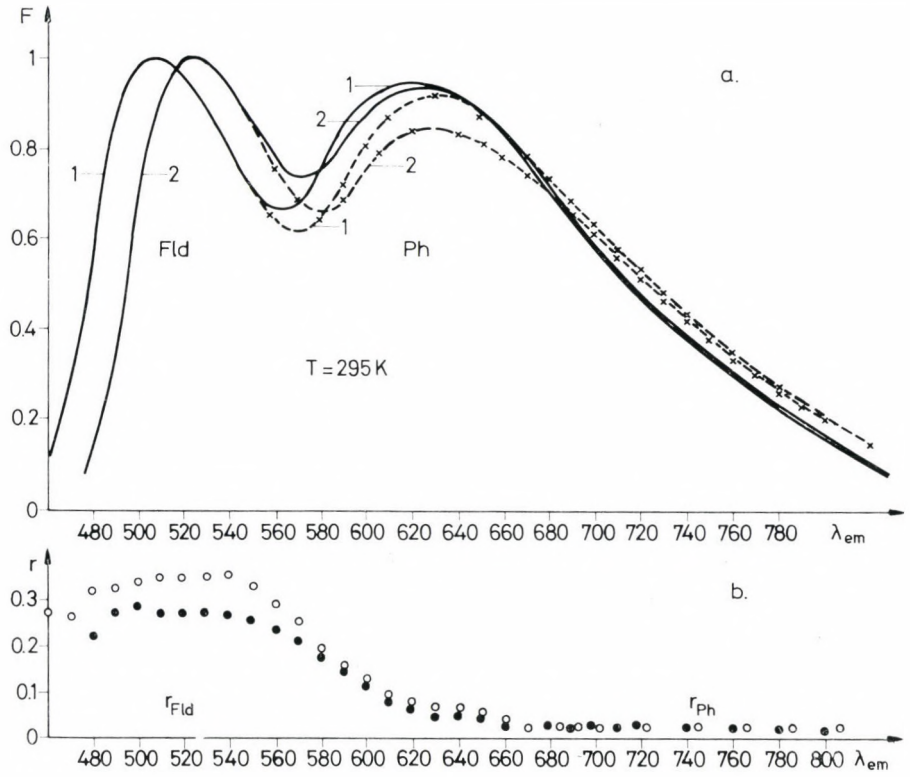

Fig. 4. Phosphorescence and delayed fluorescence of FMN in PVA matrix at 295 K for different FMN concentrations and exciting wavelengths. a) Delayed emission spectra $F(\lambda_{em})$ for concentrations $8 \cdot 10^{-5}$ g/g (1) and $4 \cdot 10^{-3}$ g/g (2), exciting wavelengths: 440 (solid line) 490 nm (dashed line); b) $r(\lambda_{em})$ spectra, exciting wavelengths: 490 nm concentrations: $8 \cdot 10^{-5}$ g/g (○○○) and $4 \cdot 10^{-3}$ g/g (●●●)

excimer may play an important role in photobiological reactions, especially in those in which electronically excited triplet states occur.

The polarized spectra of the delayed FMN emission indicate that the anisotropy of delayed fluorescence depends on the dye concentration, while the anisotropy of phosphorescence is practically independent of concentration. Similar results are obtained for polarized excitation spectra in the excitation range of the first absorption band (Fig. 3b).

The decrease of r_{Fld} at high concentration can be explained by self-quenching (Fig. 3b, $\lambda_{em} = 495$ nm) and this does not occur in phosphorescence (Fig. 3b, $\lambda_{em} = 700$ nm), therefore r_{Ph} is near zero for all concentrations and for both λ_{exc} values (Figs 2 and 3). Consequently, the phosphorescence anisotropies of the monomer and excimer at 103 and 295 K are all close to zero for the common reason mentioned above.

Acknowledgement

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Delayed Fluorescence of the Flavomononucleotide Complex

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Long-lived emission spectra and delayed fluorescence decay curves of flavomononucleotide (FMN) in poly(vinyl alcohol) film (PVA) at room temperature were measured. Pulsed N_2-laser excitation was used. It was found that the FMN complexes stabilized by PVA matrix show delayed fluorescence (band at about 540 nm) and phosphorescence (band at about 640 nm). The decay curves of the delayed fluorescence are two-exponential with decay times $\tau_1 = 62 \pm 1$ ms and $\tau_2 = 15 \pm 1$ ms.

Introduction

Flavin molecules interact with one another in the ground electronic state, and in concentrated solutions they can form intermolecular complexes: dimers or higher multimers (Jung-Do Choi, McCormick, 1980; Wehry, 1976; Nishimoto et al., 1980; Shinkai, 1978; Slifkin, 1971; Di Bartolo, 1975; Walsh, 1980). In biological systems interactions occur especially between flavin molecules stabilized by proteins i.e. between flavins and flavoproteins or/and between flavoproteins. Interaction can also occur if one of the interacting molecules is in a singlet or triplet excited electronic state (Wehry, 1976; Slifkin, 1971; Di Bartolo, 1975). It has been found that flavomononucleotide (FMN) molecules stabilized by a PVA matrix form excimers which emit relatively strong fluorescence.

The aim of this paper was to determine the spectral parameters of the FMN excimer delayed fluorescence at room temperature. The long-lived emission of the dyes is usually very weak. In order to overcome this difficulty a high-power N_2-laser was used to excite the emission.

Materials and methods

Flavomononucleotide ($C_{17}H_{20}NaO_9P$) from Fluka AG, and PVA coarse-grained powder manufactured by Loba-Chemie (Wien-Fischamend) were used for the experiments. The FMN was dissolved in an aqueous solution of PVA and

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isotropic films were obtained by water evaporation (Drabent, Olszewska, 1981). The concentrations of flavin in the matrix were: $2.4 \cdot 10^{-2}$, $1.2 \cdot 10^{-2}$, $2 \cdot 10^{-3}$ and for long-lived emission spectra even $4 \cdot 10^{-4}$ g/g (gram FMN per gram PVA).

Procedures

For measurement of the delayed fluorescence at room temperature, a laboratory-made arrangement* with pulsed laser excitation was used. The measuring set-up is shown in in Fig. 1. The N_2-laser flash illuminated the sample. The luminescence was focused on the entrance slit of a monochromator. The light passing through the monochromator was measured with a photon counter. The photomultiplier was electronically gated during the excitation. The multichannel analyser improved the signal-to-noise ratio by averaging the results of many (about 30–50) flashes. All the parts were controlled by the digital pulse generator.

a) *Delayed luminescence spectra*

The relative luminescence intensity $I(\lambda_x)/I$ (530 nm) was measured. The N_2-laser flash ($\lambda_{exc} = 337.1$ nm) photochemically damaged the sample, and therefore no site of the sample received more than 50 flashes. This measuring procedure gave correct spectra and decay curves. The difference between these curves and

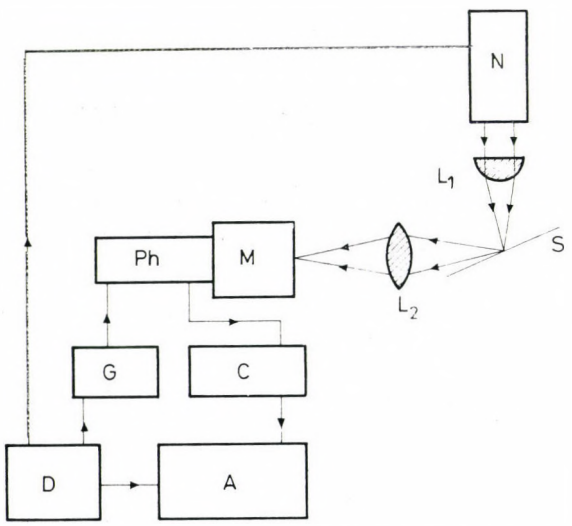

Fig. 1. Outline of the apparatus for measurement of the delayed fluorescence decay curves. N — nitrogen laser (wavelength $\lambda = 337.1$ nm, pulse energy $\simeq 200\ \mu$J, pulse width $\tau = 2.5$ ns, repetition rate 1 Hz), L_1, L_2 — lenses, S — sample, M — monochromator (Zeiss SPM-2, Si68 prism), PM — photomultiplier (EMI 9558B), G — gating unit, C — photon counter, D — digital pulse generator, A — multichannel analyser (ICA-70, KFKI, Hungary)

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those measured after the first flash was about ± 5 per cent. The intensity I (530) was measured for all sites of the sample as the reference intensity. $I(\lambda_x + 10\ nm)/I$ (530) was obtained at another site of the sample. Points of the spectra represent the integrated delayed luminescence intensity $I(\lambda_x)$ in the time range 1–11 ms after excitation. All spectra were corrected for the spectral sensitivity of the equipment and for the background light.

b) *Kinetics of the decay*

The decays are given in the time range 1–100 ms (excitation at $t = 0$) with a time resolution of 1 ms. We did not find any faster delayed fluorescence component, even when we used the maximum time resolution (4 μs/channel) of our instrument. The decay was measured at $\lambda_{em} = 530$ nm, the spectral bandwidth was 10 nm and 100 decays were averaged.

c) *Excitation spectra*

The excitation spectra were measured with a Perkin–Elmer MPF-44A spectrofluorimeter. Two types of excitation spectra were measured: at $\lambda_{em} =$ -490 nm (the region of fluorescence predominantly relating to the FMN monomer) and at $\lambda_{em} = 570$ nm (mainly the emission of the excimer). The study was made at four dye concentrations.

All measurements were carried out at room temperature. All samples were protected from light.

Results and discussion

1. Excitation spectra in the region around 337 nm

The long-lived fluorescence was excited at $\lambda_{exc} = 337$ nm. To determine the efficiency of the excimer formation processes in this excitation region, the fluorescence excitation spectra at two emission wavelengths (570 nm and 490 nm) and at several FMN concentrations were measured. The results obtained with the lowest and the highest FMN concentrations are shown in Fig. 2 (curves 1–4). The difference excitation spectrum $E_d(\lambda_{exc})$ (Fig. 2b) for $\lambda_{em} = 570$ nm (the excimer fluorescence region) was calculated from the equation

$$E_d = E_{exc1} - k \cdot E_{exc2}$$

where k is the coefficient which normalizes the excitation spectrum for the lowest concentration (E_{exc2}, Fig. 2a, curve 2) to that for the highest concentration (E_{exc1}, Fig. 2a, curve 1). The coefficient k was calculated for λ_{exc} at about the maximum of absorption band II, to obtain $E_d = 0$ for $\lambda_{exc} = 360$ nm. The results in Fig. 2b indicate that the efficiency of excimer formation at $\lambda_{exc} = 337$ nm is higher than at $\lambda_{exc} = 364$ nm, which is the maximum of absorption band II. Thus, excitation at 337 nm can be used to study FMN excimer emission.

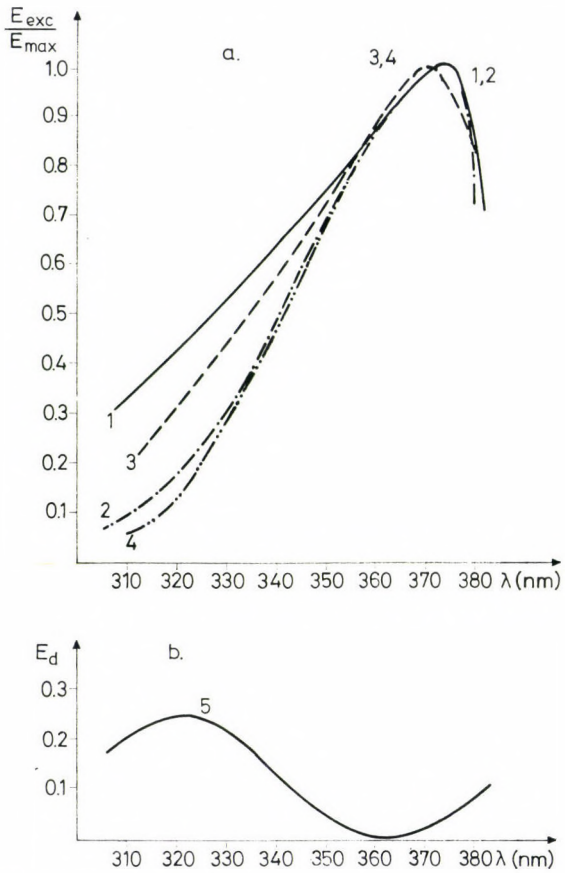

Fig. 2. Fluorescence excitation spectra of the flavomononucleotide (FMN) in a PVA matrix in the region of 337 nm for two dye concentrations and different wavelengths of emission λ_{em}. a) Curve 1 – c = $2.4 \cdot 10^{-2}$ g/g, $\lambda_{em} = 570$ nm, E_{exc1}; curve 2 – c = $4 \cdot 10^{-4}$ g/g, $\lambda_{em} = 570$ nm, E_{exc2}; curve 3 – c = $2.4 \cdot 10^{-2}$ g/g, $\lambda_{em} = 490$ nm; curve 4 – c = $4 \cdot 10^{-4}$ g/g, $\lambda_{em} = 490$ nm. b) Curve 5 — difference excitation spectrum $E_d = E_{exc1} - kE_{exc2}$, where k is the normalizing coefficient for $\lambda_{exc} = 360$ nm

2. Delayed fluorescence and phosphorescence spectra

The long-lived emission spectra at four different concentrations of FMN in PVA films are shown (in arbitrary units) in Fig. 3 (curves 1). The long-lived emission spectrum $F(\lambda_{em})$ measured at room temperature is a superposition of the delayed fluorescence and phosphorescence of the FMN monomer and excimer:

$$F(\lambda_{em}) = F_d(\lambda_{em}) + F_{ph}(\lambda_{em}) \tag{1}$$

where: $F_d(\lambda_{em})$ = delayed fluorescence spectrum of monomer and excimer, and $F_{ph}(\lambda_{em})$ = phosphorescence spectrum of monomer and excimer.

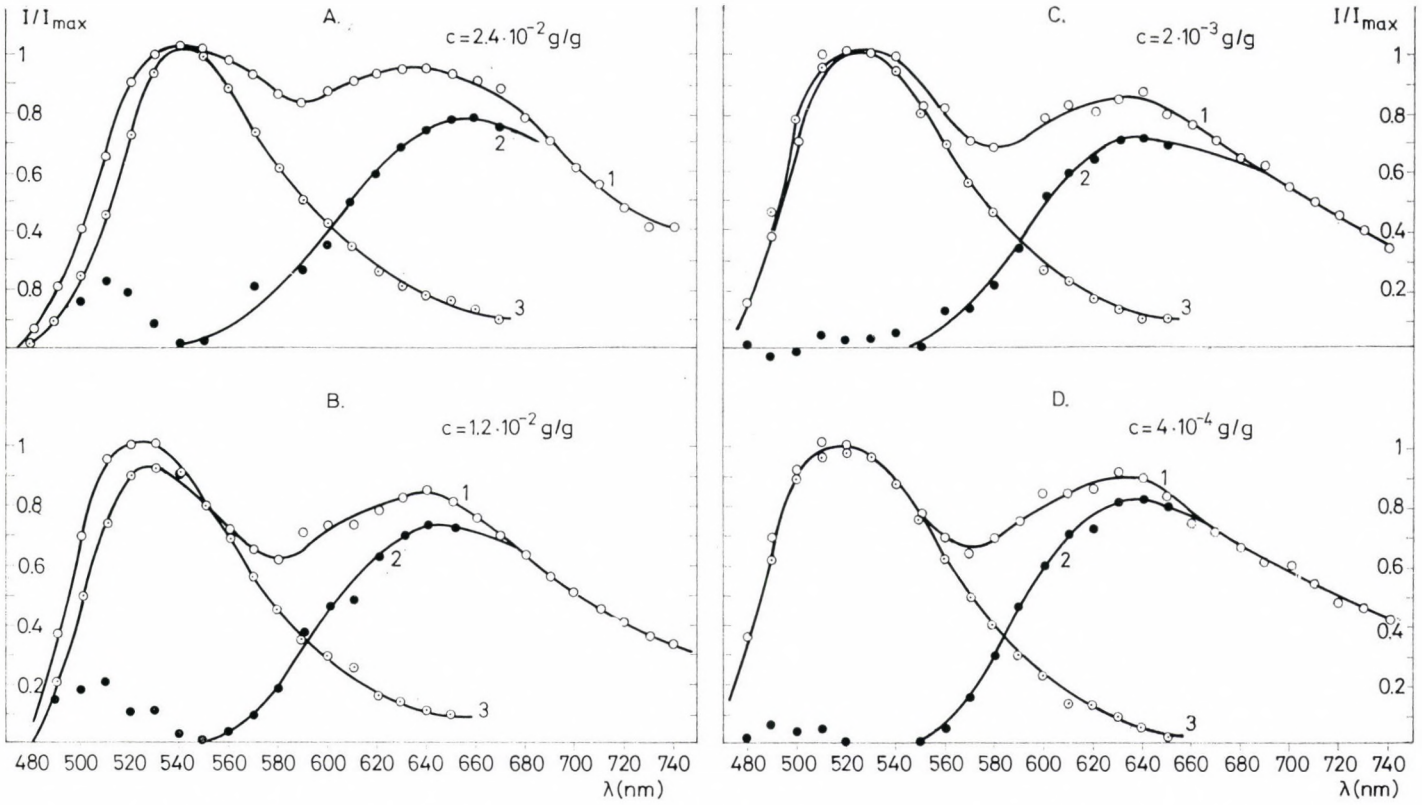

Fig. 3. Delayed fluorescence spectra and phosphorescence spectra of the flavomononucleotide (FMN) in a PVA matrix for different dye concentrations. Curve 1 — long-lived emission (in relative units), curve 2 — phosphorescence spectra after separation, curve 3 — normalized fluorescence spectra, $bF_f(\lambda_{em})$

The distribution of the transition probability of the delayed fluorescence can be approximated by that of the prompt fluorescence $F_f(\lambda_{em})$ for the same dye concentration. Therefore, after normalizing both spectra to the maximum, $F_d(\lambda_{em}) = F_f(\lambda_{em})$. The prompt fluorescence spectra of the samples used in this experiment are known from our previous measurements. The phosphorescence spectra can therefore be separated:

$$F_{ph}(\lambda_{em}) = F(\lambda_{em}) - bF_f(\lambda_{em}) \tag{2}$$

b was calculated for each dye concentration.

The results of these calculations are shown in Fig. 3 (curves 2). The phosphorescence of the FMN in the PVA matrix at room temperature was a large band with λ_{max} at about 640 nm.

It should be noted that, for a relatively high excimer concentration (Fig. 3 A, B), an emission band appears at about 510 nm in the spectra calculated from eq. (2). This is the region of the monomer fluorescence. The weak band in the monomer fluorescence region indicates that the quantum yield of the delayed fluorescence compared to the prompt fluorescence is higher for monomer than for excimer.

3. Lifetime τ of the FMN delayed fluorescence

The decay curves were measured at $\lambda_{em} = 530$ nm. This is the spectral region of the FMN delayed fluorescence band, and these are therefore the decay curves of the delayed fluorescence. The measurements were made at several FMN concentrations. The curves obtained were similar, and we shall discuss only the decay curve for the FMN concentration $1.2 \cdot 10^{-2}$ g/g (Fig. 4, curve 1). As may be seen in Fig. 4a, the logarithmic decay curve (curve 2) displays a significant deviation from linearity. The experimental decay curve $I(t)$ is thus multiexponential, and the emission is due to two or more kinds of emission centres with different lifetimes. On the other hand, curve 2 is linear in the range 70–100 ms. The delayed fluorescence in this range is therefore due to only one type of emission centre. The intensity $I_1(t)$ of the delayed fluorescence of these centres can be written as

$$I_1(t) = I_{01}\, e^{-t/\tau_1}$$

The decay time τ_1 can be calculated as

$$\tau_1 = -\, 1/\tan \alpha_1$$

from curve 2 (Fig. 4) in the time range 7–100 ms. An average value of $\tau_1 = 62 \pm 1$ ms was obtained. The value I_{01} can also be determined from this curve. The decay curve I_1 can therefore be calculated in the entire time range (Fig. 4b, curve 3). It was found that the decay curve

$$I(t) - I_1(t) = I_2(t)$$

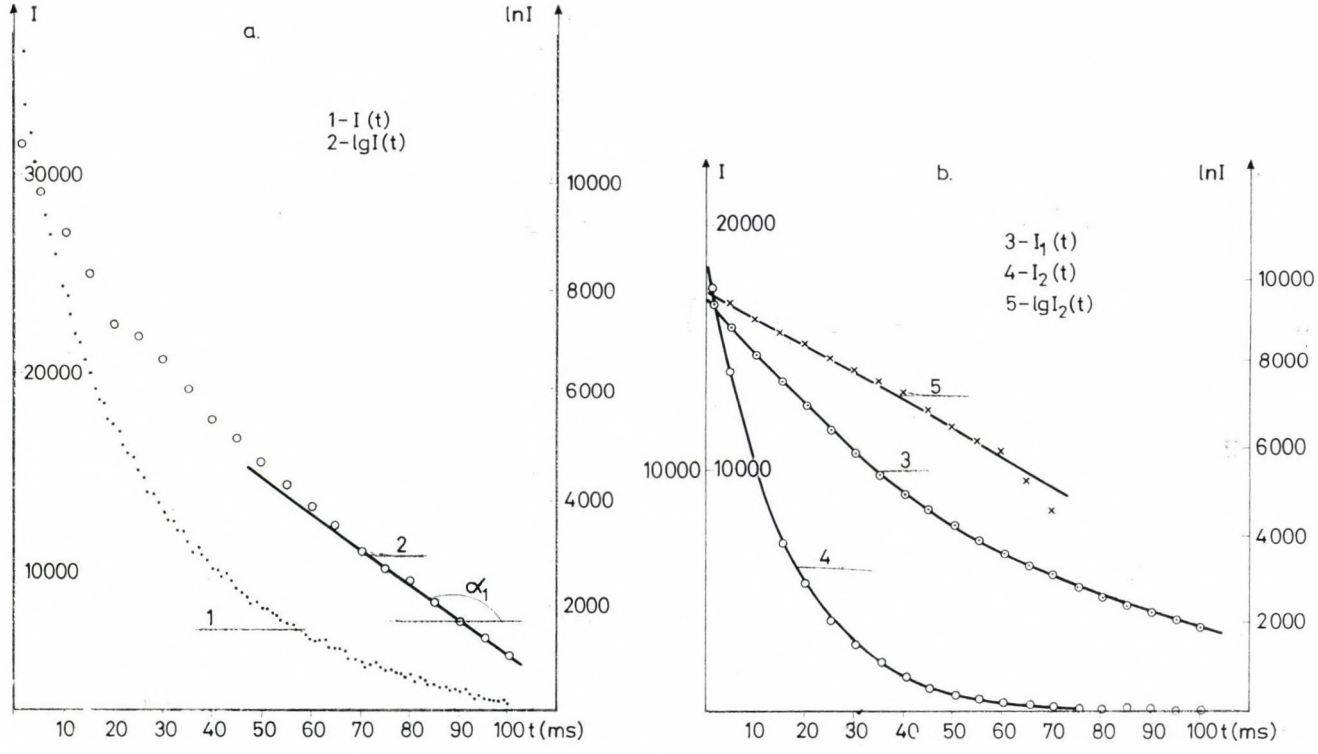

Fig. 4. Decay curves of the FMN delayed fluorescence in the PVA matrix for $\lambda_{em} = 530$ nm. a) Curve 1 — decay curve of the FMN delayed fluorescence $I(t)$, curve 2 — ln $I(t)$; b) curve 3 — fluorescence decay curve of the first kind of emission centre, $I_1(t)$, curve 4 — fluorescence decay curve of the second kind of emission centre, $I_2(t)$, curve 5 — ln $I_2(t)$

is also a single exponential curve (Fig. 4b, curves 4 and 5). The decay time of the delayed fluorescence I_2 calculated from curve 5 is $\tau_2 = 15 \pm 1$ ms.

This separation of the experimental curve onto two exponential decay curves and calculated decay times τ_1 and τ_2 indicates that the decay times of the delayed fluorescence of the FMN excimer and monomer are different.

In conclusion, the FMN complexes stabilized by PVA matrix exhibit delayed fluorescence (band about 540 nm) and phosphorescence (band about 640 nm) at room temperature. The decay curves of the delayed fluorescence are two-exponential with decay times $\tau_1 = 62 \pm 1$ ms and $\tau_2 = 15 \pm 1$ ms. This indicates that the lifetimes of the delayed fluorescence of the FMN excimer and monomer are different.

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Morphological Comparison of Synaptosome Preparations

(Short Communication)

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The synaptosome preparation offers an opportunity to obtain the synaptic membrane in large quantity by a relatively simple procedure. However, the surveyor — in studying its behaviour — needs information about the physiological properties and the morphological composition of the preparation. Having in mind to find a good object for biophysical measurement we have prepared synaptosomes by three different methods and compared their biochemical, biophysical and morphological properties. The biochemical and biophysical characterizations were already reported (Hargittai et al., 1982), the present notes contain the details of their morphology.

Rat cortical synaptosomes were prepared according to two methods, one of which uses slightly hyperosmotic sucrose media (Gray, Whittaker, 1962; Hajós, 1975) while the other is the isoosmotic procedure of Booth and Clark (1978). The final synaptosomal fraction was kept at 4 °C before use.

The electron microscopic examination was performed to check the structural integrity and purity of the synaptosome preparations following the method of Nagy et al. (1977). In brief: the synaptosomal pellet was resuspended in Krebs solution (145 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 2.0 mM NaH_2PO_4, 10 mM glucose, 20 mM Tris-base; pH adjusted to 7.4 with maleic acid) and allowed to stand at 37 °C for 10 minutes. After this incubation the suspension was diluted 2-fold with Karnowsky fixative and centrifuged in an Eppendorf desk-top centrifuge for 10 minutes, to obtain pellets for osmic acid post-fixation. The samples were dehydrated in alcohol and embedded in Durcupan. The sections were cut from the middle part of the samples and were examined with a JEOL 100 B electron microscope.

The electron microscopic examination revealed that the synaptosomes (Fig. 1 A, B, C) were similar to those observed by others (Booth, Clark, 1978; Gray, Whittaker, 1962; Hajós, 1975). There were no significant differences be-

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tween the electron micrographs of the three differently prepared synaptosomal fractions after their incubation in Krebs solution at 37 °C for 10 minutes. In all cases, the synaptosome content, estimated according to the number of vesicle-containing particules, proved to be larger than 75 per cent.

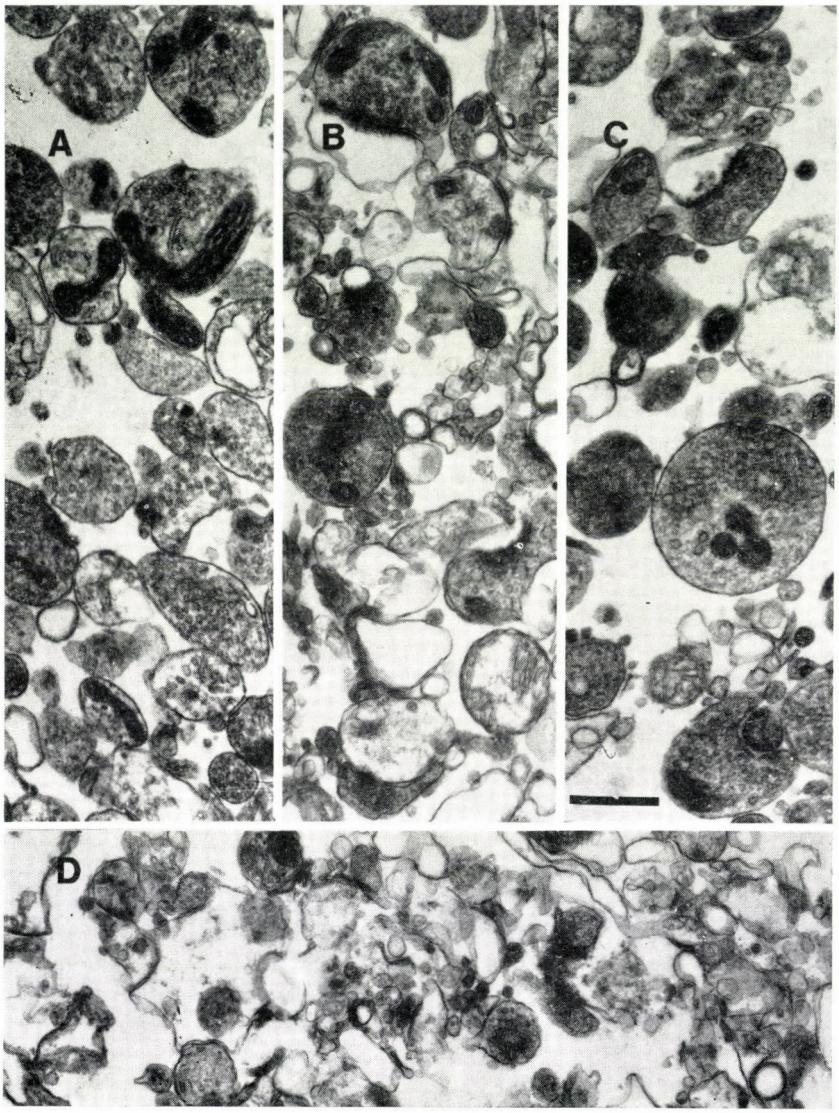

Fig. 1. Electron microscopic pictures of different synaptosomal preparations. Synaptosome fraction prepared by the method of Gray and Whittaker (A), by that of Hajós (B) and by that of Booth and Clark (c). Fig. 1D shows a synaptosome suspension prepared by the Hajós method which was damaged by the osmotic shock. The bar represents 0.5 μm

Most properties of the synaptosome preparations were preserved in physiological saline, regardless the technique of preparation. During the preparation, the synaptosomes can be subjected to osmotic shock, such as a sudden dilution of the hyperosmotic separating sucrose solution. Therefore, in our studies, when diluting the hyperosmotic solution the necessary amount of distilled water was gradually added, over 30 minutes, at 0 °C while the suspension was gently stirred. Figure 1 D displays a synaptosomal preparation: damaged, slightly vacuolised by rapid dilution of the synaptosomal fraction obtained from a gradient as described by Gray and Whittaker (1962).

Although there is a great difference in osmolarity of the separating media, our three synaptosomal fractions do not differ from each other regarding their electron micrographs. The results are in good agreement with the physiological characterization of synaptosome fractions and the enzyme activity criteria (Hargittai et al., 1982).

Acknowledgement

We thank L. Keszthelyi for the critical comments on the manuscript and F. Joó for valuable help during the preparation of electron microscopic sections.

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Intermediate and Zigzag (Electronic) States (IS and ZZS) and Quantum Size Effects (QSE) in Finite Crystals and Biopolymers: Proposals for Experiments[1]

(Short Communication)

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Recent results of QSE experiments suggest that the ISs might be observable, since they belong to the class of one-electronic QSE states that have already been observed. Therefore, slightly modified tunnelling experiments (Jaklevic, Lambe, 1972; Lutskil, 1970) as well as surface sensitive optical absorption (Fuchs, Kliever, 1969) and photoelectric yield measurements (Anderegg et al., 1971) are proposed for studying the hypothetic ISs in inorganic and organic insulators and semiconductors such as certain oxides, molecular crystals of nucleotide bases, graphite and its intercalation compounds, and layers of oriented lengthy homopolynucleotides and proteins.

Theory

It was Cottey (1971, 1972) who first treated correctly the band edge state (BES) solutions of the Schrődinger equation stated for both infinite and finite one-dimensional periodic potentials. The BESs form a special class of the ISs (Biczó, 1977; 1978; Biczó. Lukovits, 1979). The ZZS, as a generalization of the IS, has been introduced in the latter paper, where its possible role has also been outlined in certain processes of molecular biology. The ZZS may represent a very well controlled pathway of the electrons.

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Experiment

Another study of Cottey (1975) strongly supports that certain observed structures in the tunnelling current through an oxide layer into a thin metal layer (for Pb: Lutskil, 1970; Jaklevic et al., 1971; for Mg: Jaklevic, Lambe, 1972) are due to the one-electronic QSE levels. Naturally, some of them may belong to the ISs. There are further evidences, obtained for potassium films of different thicknesses, for the observability of such levels: the QSE is manifested in well observable shoulders in the absorption spectra (for thickness of 46 Å: Fuchs, Kliever, 1969; for 27 Å: Mukhopadhyay, Lundqvist, 1978, 1980), as well as in characteristic structures in the photoelectric yield spectra (for 27, 58 and 100 Å: Anderegg et al., 1971). These are in good concordance with the most recent theoretical QSE studies (Apell, Ahlqvist, 1981; Ahlqvist, Apell, 1981; Ahlqvist, 1982).

The proposed experiments

(I) Slightly modified Lutskil and Jaklevic, Lambe, Mikkor, Wassell (Lutskil, 1970; Jaklevic et al., 1971) experiment. We propose to sharp point the measurement to the behaviour of the thin oxide layer rather than to that of the metallic one: a broad-energy-band insulator layer has to be investigated which should be placed in between two metal contacts having variable Fermi levels. If the available bias is not sufficiently high for allowing to study the entire actual energy interval, different metals must be applied as contacts. Arriving with the Fermi levels at the band edges or at the resonant sites (Biczó, 1978) of the insulator's energy bands, we can expect an increase of the tunnelling current by the turning over (state pump) process (Biczó, Lukovits, 1979). It is probable that the application of an a.c. bias may significantly increase the sensitivity of the measurements by generating resonant oscillations. The application of an insulating (and not a conducting) layer, having possibly perfect crystal structure, is very important to avoid the depression of the just studied "enhanced tunnelling" by other stronger competitive conduction phenomena.

(II) Slight variations of the surface parameters, e.g. outer electric or magnetic fields or surface covering, may induce a turning over of the ISs (Biczó, Lukovits, 1979, 1981) in a thin film. At the same time, the real bulk-like states are generally stable against such outer effects. This sensibility of the ISs, namely, the sudden appearance and disappearance of their energy levels in the QSE spectrum under the effect of the outer field, may be an acceptable proof for their existence, when the QSE spectra are observed by surface (and not bulk!) sensitive optical absorption and photoelectron yield techniques.

(III) The study of graphite and its intercalation compounds would also be very instructive. To proceed towards the biological problems, we propose to perform the above mentioned experiments not merely for inorganic, but also for organic insulators and semiconductors such as certain molecular crystals of

nucleotide bases, as well as for layers of oriented lengthy homopolynucleotides and proteins. These are not intrinsic semiconductors because of their very large forbidden energy gap but, at the same time, they have sufficiently strong longitudinal interactions for appropriate electronic delocalization (Ladik, 1982, and refs therein).

We quote our most recent paper (Biczó, 1983) concerning the relation of the ISs and the long distance electron transport processes of molecular biology (Larsson, 1982).

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The Effect of External Sodium on Ouabain-insensitive K Influx in Fresh Human Red Blood Cells

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The rate of ^{42}K influx was investigated at various external sodium and ouabain concentrations in human red blood cells.

In agreement with earlier reports, in red blood cells not treated with ouabain, Na did not affect K influx when $[K]_0$ was 5.0 mM while it reduced the influx at $[K]_0 = 0.15$ mM. However, Na stimulated ^{42}K influx at both 5.0 mM and 0.15 mM in cells treated with ouabain (1×10^{-5} M). When external Na concentration was raised from 0 to 72 mM the rate of ^{42}K influx increased at $[K]_0 = 0.15$ mM and at ouabain $= 1 \times 10^{-5}$ M. The effect of external Na at different ouabain concentrations showed that K influx was inhibited by Na without or with ouabain in less than 5×10^{-6} M while an increased K influx could be observed with higher ouabain concentrations in the incubation media.

The results suggest that in the case of the complete inhibition of ouabain-sensitive K influx the electrochemical gradient of the Na ions may serve as a driving force for the inward movement of potassium.

Introduction

It is generally considered that at least three conceptually distinct membrane pathways are available for the movement of cations in human red blood cells (Hoffman, Kregenow, 1966; Sachs, 1971). Thus ions can move by an active Na–K pump specifically inhibited by ouabain (Schatzmann, 1953; Kahn, Acheson, 1955; Glynn, 1957), by the ouabain-insensitive carrier mediated transport and via passive diffusion (Gárdos, 1964; Hoffman, 1966; Funder, Wieth, 1967; Lubowitz, Whittam, 1969; Sachs, 1970; Beaugé, 1975; Lew, Beaugé, 1979).

Simple diffusion is thought to have only a negligible role in the cation transport because of the very low cation permeability of the erythrocyte membrane (Solomon, 1952; Glynn, 1956; Sachs, 1977) while ouabain-sensitive Na–K pump has a major importance and its characteristics have been widely studied in the last two decades (Gárdos, 1964; Garrahan, Glynn, 1967a; Garrahan, Glynn, 1967c; Garrahan, Glynn, 1967d; Glynn, Karlish, 1976; Garrahan, Glynn, 1967e;

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Heller, Beck, 1978). The interactions and the effects of external alkali metal ions with the Na–K pump have also been described in detail (Sachs, 1974; Hobbs, Dunham, 1976; Kropp, Sachs, 1977; Hobbs, Dunham, 1978). It is known that at physiological (5 mM) external potassium concentration $[K]_0$, potassium influx is rather insensitive to external sodium concentration $[Na]_0$ but at low potassium concentrations even low levels of sodium inhibit potassium influx (Garrahan, Glynn, 1967b).

Little attention had however been devoted to the Na and K fluxes in human red blood cells which are not inhibited by ouabain. Hoffman and Kregenow (1966) assumed the existence of a ouabain-insensitive, ethacrynic acid-sensitive sodium pump inactive at 30 mM $[Na]_0$ but fully active at 150 mM or higher $[Na]_0$. Lubowitz and Whittam (1969) suggested that these ouabain-insensitive Na movements represent a Na-Na exchange.

This paper describes the sensitivity of the ouabain-insensitive K influx to external K in low potassium media. The concentration dependence of ouabain-inhibition with and without external Na at low $[K]_0$ was also studied.

Materials and methods

Solutions and Chemicals

"Choline solution" (mM): Choline-Cl 145; $CaCl_2$ 2.5; $MgCl_2$ 2.0; Tris-HCl (pH: 7.4 at 37 °C) 5.0; glucose 10. "Radioactive solutions" (mM): KCl 5.0; or 0.15; NaCl 140; or 145; $CaCl_2$ 2.5; $MgCl_2$ 2.0; Tris-HCl (pH: 7.4 at 37 °C) 5.0; glucose 10.0. In solutions of different NaCl and KCl concentrations isotonicity was guaranteed by adding choline-Cl in proper amounts and controlled by means of "Knauer Halbmikro Osmometer". ^{42}K was obtained as a sterile isotonic solution of KCl from the Isotope Institute of the Hungarian Academy of Sciences. Ouabain was obtained from Serva (Heidelberg), and dissolved in the appropriate salt solution on the day of use with the aid of ethanol. The ethanol concentration both in "choline" and "radioactive" solutions was less than 0.5 per mille.

Experimental procedures

Venous blood from healthy human donors was drawn into heparinized syringes. Cells were prepared for the experiments by washing them 4 times (3000 g; 5 min; 18 °C) in 20 volumes of "choline solution" and suspending them in the same media at twice the final haematocrit (5.0 per cent).

Erythrocyte suspension was distributed among plastic centrifuge tubes (2–2 ml) and ouabain in the desired concentrations was added to them. Experiments were started by transferring the tubes to a bath at 37 °C. After 10 min "preincubation period" the suitable radioactive media of the same volume (2 ml) was added to each suspension by means of an automatic "Oxford" micropipette, having a time delay between the first and the last tube less than 2 min. ^{42}K up-

take periods were 30, 60, 90 and 120 minutes, respectively. Since potassium uptake in the presence, as well as in the absence of external sodium, was found to be linear with time for at least 2 hrs, therefore influx was estimated by measuring the differences in the uptakes of ^{42}K in the 90th and the 30th minutes. At the end of the incubation period the same washing procedure, as described above was carried out. The washing solution removed after the last centrifugation, contained no detectable radioactivity. The washed cells were then lysed in redistilled water (5 ml), and the activities of the haemolysates were determined by means of a conventional well-type NaI/Tl crystal scintillation counter (Gamma, type NK-350).

Haematocrits were determined by spinning for 20 min at 3000 g in normal haematocrit tubes using at least 4 parallel samples. K influx ($\mu mol \times l^{-1} \times RBC \times \times h^{-1}$) was calculated from the relative specific activity of the radioactive solution and from the haematocrits. At least 3 parallel tubes were incubated in all of the experiments and results were rejected when the influx in any of the three samples deviated from mean by more than 5 per cent.

Results

Figure 1 shows ^{42}K influx of red blood cells not treated with ouabain as a function of external sodium concentration. At $[K]_0 = 5$ mM the value of K influx was about 1.4 $mmol \times l^{-1} RBC \times h^{-1}$ and it remained on the same level when Na concentration was increased from 0 to 67 mM in the incubation media (Fig. 1a). However, at low (0.15 mM) $[K]_0$, external sodium ions decreased K influx to one fifth (Fig. 1b). The decreasing effect of $[Na]_0$ showed a concentration dependence.

In the following experiments the effect of external sodium ions on K influx was investigated in cells treated with 1×10^{-5} M ouabain, i.e. with the concentration at which the maximal inhibitory effect on Na–K pump of the drug developed in the presence of Na (see Fig. 4). As shown on Fig. 2 there was an increase in the rate of K uptake in the solution containing Na both at 5 mM $[K]_0$ (a) and at 0.15 mM $[K]_0$ (b), respectively, as compared to the Na-free "choline solution". On the basis of the values of K uptake in the period of 30 to 120 min, when the uptake curve was linear with time, the calculated rate of K influx was:

at 0.15 mM $[K]_0$ in Na-free "choline solution"
$10.9\ \mu mol \times l^{-1}\ RBC \times h^{-1}$
at 0.15 mM $[K]_0$ Na-containing solution
$15.4\ \mu mol \times l^{-1}\ RBC \times h^{-1}$
at 5.00 mM $[K]_0$ in Na-free "choline solution"
$117.3\ \mu mol \times l^{-1}\ RBC \times h^{-1}$
at 5.00 mM $[K]_0$ Na-containing solution
$309.1\ \mu mol \times l^{-1}\ RBC \times h^{-1}$

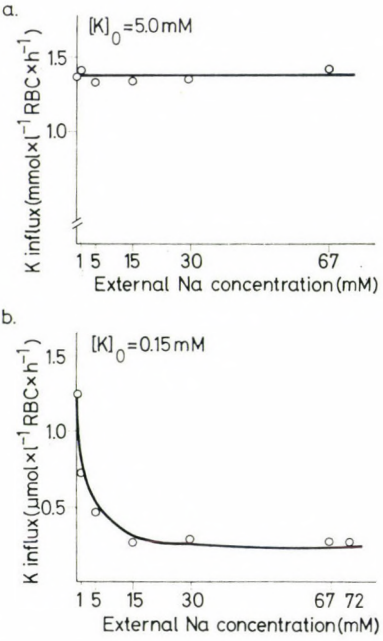

Fig. 1a and b. The effect of $[Na]_0$ on ^{42}K influx in red blood cells at $[K]_0 = 5$ mM (*a*) and at $[K]_0 = 0.15$ mM (*b*). Isotonicity was assured by choline (see text). RBC = red blood cells. K influx was estimated from the uptake of ^{42}K in 30 to 120 min uptake periods

Paradoxically, while K influx in 30 to 120 min period was promoted by Na ions in the incubation media in ouabain-treated cells, an inhibition of K entrance by extracellular Na ions was detectable in the first 15 min of ^{42}K influx.

In the further experiments we used the 30 to 90 min uptake period, which was linear and long enough for the full development of the sodium ions' effect. The ouabain concentration was the same as above and the external Na concentration was raised gradually from 0 to 72 mM. Figure 3a is a plot of the rate of ouabain-insensitive K influx as a function of the external concentration of sodium at 5 mM $[K]_0$ while in Fig. 3b the rate of ouabain-insensitive K influx is plotted against $[Na]_0$ at 0.15 mM $[K]_0$. At $[K]_0 = 5$ mM the K influx at 0.5–5 mM $[Na]_0$ was not statistically significantly different if the $[Na]_0$ values were between 0.5 and 5 mM, while between 5 and 67 mM $[Na]_0$ values the difference was statistically significant ($p < 0.01$). At $[K]_0 = 0.15$ mM statistically significant difference ($p < 0.05$) could be detected only between 5 and 72 mM $[Na]_0$. The results of these experiments suggest that the ouabain-insensitive component(s) of K influx is (are) promoted by external Na both at physiological (5 mM) and at low (0.15 mM) $[K]_0$.

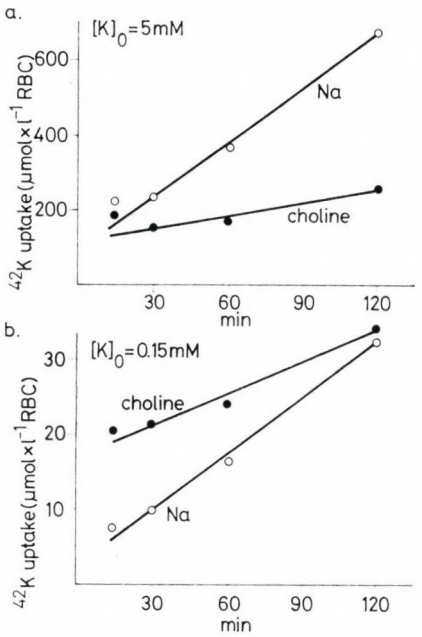

Fig. 2a and b. The effect of $[Na]_0$ on ^{42}K uptake in ouabain treated red blood cells (*a*) ○——○ $[K]_0 = 5$ mM, $[Na]_0 = 67$ mM, $[choline]_0 = 72$ mM. ●——● $K_0 = 5$ mM, $[choline]_0 = 139$ mM. (*b*) ○——○ $[K]_0 = 0.15$ mM, $[Na]_0 = 72$ mM, $[choline]_0 = 72$ mM. ●——● $[K]_0 = 0.15$ mM, $[choline]_0 = 144$ mM. Ouabain (1×10^{-5} M) was administered simultaneously with ^{42}K to the uptake solution

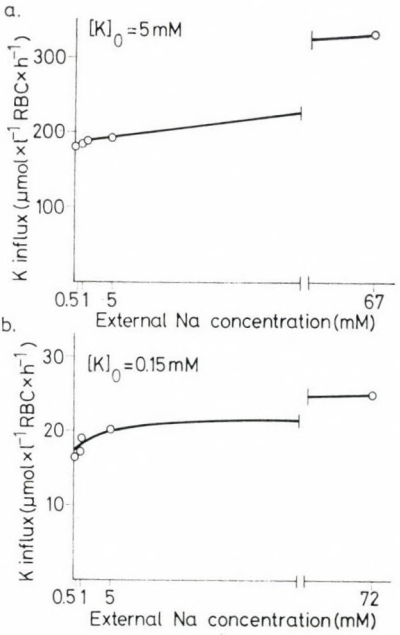

Fig. 3a and b. The effect of $[Na]_0$ on ^{42}K influx in ouabain treated red blood cells. Ouabain (1×10^{-5} M) was added the cell suspension 10 min prior to the influx period. (*a*) $[K]_0 = 5$ mM, $[Na]_0 = 67$ mM, $[choline]_0 = 72$ mM. (*b*) $[K]_0 = 0.15$ mM, $[Na]_0 = 72$ mM, $[choline]_0 = 72$ nM

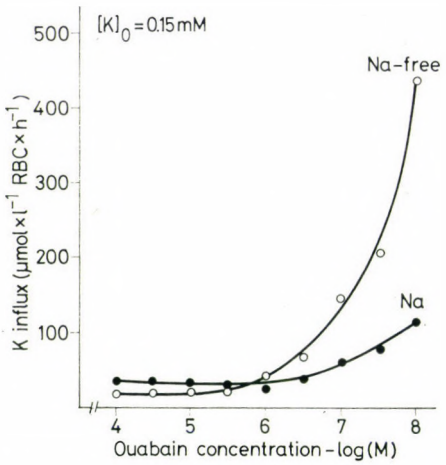

Fig. 4. Concentration dependence of ouabain inhibition on ^{42}K influx in the presence and absence of external sodium ●——● $[K]_0 = 0.15$ mM, $[Na]_0 = 72$ mM, $[choline]_0 = 72$ mM. ○——○ $[K]_0 = 0.15$ mM, $[choline]_0 = 144$ mM. Cells were pretreated with ouabain 10 min prior to the influx period

It seemed worth to study the concentration dependence of the inhibitory effect of ouabain in the presence and in the absence of external sodium ions at $[K]_0 = 0.15$ mM. K influx was inhibited by external Na in solutions containing ouabain below 5×10^{-6} M, while a $[Na]_0$-stimulated K influx could be observed when ouabain concentration was above 1×10^{-5} M, as compared to the influx in Na-free solution in the presence of similar ouabain concentrations (Fig. 4).

Discussion

The major part of Na and K movement across red blood cell membrane is mediated by an ouabain-sensitive Na–K pump (Schatzmann, 1953; Kahn, Acheson, 1955; Glynn, 1957), and can be inhibited by external sodium ions at low potassium concentrations (Garrahan, Glynn, 1967b). However, with 5 mM $[K]_0$, potassium influx is rather insensitive to $[Na]_0$ and even high levels of external Na do not inhibit (Garrahan, Glynn, 1967b; Sachs, 1977). Our results shown in Figs 1 *a* and *b* are in good agreement with these observations and can be sufficiently explained by the competition of sodium and potassium ions for the K carrier binding sites (Glynn, Karlish, 1976; Hobbs, Dunham, 1978).

Ouabain-insensitive K, Na fluxes were demonstrated by several authors (Hoffman, 1966; Hoffman, Kregenow, 1966; Funder, Wieth, 1967; Lubowitz, Whittam, 1969; Beaugé, 1975). Various mechanisms were supposed to be responsible for these fluxes; exchange diffusion (Ussing, 1949; Lubowitz, Whittam, 1969),

active transport by a ouabain-insensitive "pump II" (Hoffman, 1966; Hoffman, Kregenow, 1966), and downhill-leak of ions (Hoffman, 1966).

Hoffman and Kregenow described a K influx depending upon the presence of external sodium (1966). It was glycoside insensitive but sensitive to ethacrynic acid. A support came from the work of Lubowitz and Whittam (1969), who found an increase in K influx in ouabain treated red blood cells in the presence of external sodium. This increase, however, was hardly significant and an increase, though not significant, could be observed also under control conditions.

In nystatin treated low K, low Na and Li loaded red blood cells Wiley and Cooper described a ouabain insensitive, furosemide sensitive Na–K cotransport (1974).

Based on their recent experimental findings Sarkadi et al. constructed a hypothetical model in which a furosemide or phlorethin sensitive carrier mediates the inward movement of Na and Li in an energy expenditure of downhill outward Na or Li movement (1978).

In our experiments ouabain was used in a concentration of 1×10^{-5} M since at this concentration ouabain receptor sites of human red blood cell membrane are saturated and the inhibitory effect is complete (Heller, Beck, 1978).

As shown in Figs 3 and 4, we observed a marked increase of K influx in Na containing solutions as compared to the Na-free solutions. In these experiments cells had been treated for 10 min with ouabain before they were moved to the radioactive solution. The increase did not depend on whether physiological (5.0 mM) or low (0.15 mM) external K concentrations were used.

Martin described an effect of external cations on choline transport (1972). However, in our experiment a direct effect of choline on K transport at various external potassium concentrations could be excluded since choline was present both in Na-free and in Na-containing medias.

In some experiments ouabain and radioactive potassium were added to the cells simultaneously. From the data presented on Figs 2 a and b, one can see that the increasing effect of external Na ions on K influx comes into operation only after a delay, i.e. if ^{42}K and ouabain are added at the same time; the initial difference (K influx in Na-free solution is three times higher than that in Na-containing media) masks the effects of $[Na]_0$ on potassium movement.

The main conclusion of the present study is that after the full inhibition of ouabain-sensitive potassium transporting mechanism, the electrochemical gradient of the sodium ions may serve as a driving force for K influx.

Acknowledgements

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Comparative Studies on Primycin and Gramicidin Induced Cation Transport Changes in Human Erythrocytes

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A comparative study of the actions of antibiotics primycin and gramicidin on tracer exchange of red blood cells at steady state salt distribution was carried out. The equilibrium radioactive ion distribution was not achieved at definite concentrations of either antibiotics. A development of Adam's statistical mechanical model for cooperative transitions in membranes are presented to explain the results obtained.

Introduction

The understanding of the mechanism of the effect of biologically active compounds is important for the pharmacist because it helps the proper synthesis of these materials. Some of these compounds show membrane active properties inducing permeability changes in cell membranes. This is why the action of these chemicals on membrane permeability may be used as a test of their biological activities. On the other hand by these studies one can get a better insight into the membrane structure and function.

Antibiotic primycin (Vályi-Nagy et al., 1954; Aberhart et al., 1970; Aberhart et al., 1974) is one of the biologically active substances that can change the ion permeability of both cells and bilayer lipid membranes. The amphiphilic molecule consists of a lactone ring having a sugar and a guanidino group. By means of the latter group it has a positive charge. Primycin has activity against Gram positive bacteria and accelerates wound healing. In some biochemical studies it was found that the bacteriostatic action of primycin is based on its interaction with the membranes (Horváth et al., 1979; Mészáros et al., 1979; Kövér et al., 1976).

Recently it has been shown (Blaskó et al., 1979; Blaskó, Györgyi, 1980; Blaskó, Györgyi, 1981) that primycin increases the permeability of erythrocytes

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to alkali cations according to the sequence $Cs^+ > Rb^+ \sim K^+ \gg Na^+$. It also increases the conductance of bilayer lipid membranes, in these model membranes, however, another sequence of ions was found: $Na^+ > K^+ \sim Rb^+ > Cs^+$. The conductance of bilayer membrane shows a second order function on antibiotic concentration. In our preliminary experiments on phosphatidylserine bilayer membranes single channel conductivity has been measured in the presence of primycin.

One of the well known channel forming antibiotics, gramicidin has similar properties on model membranes (Goodall, 1970; Urry et al., 1971; Bamberg, Lauger, 1974; Veatch et al., 1974; Hladky, Haydon, 1972; Schagina et al., 1978). The question has arisen whether these similarities exist on biological membranes, too. In our recent studies on red blood cells (Blaskó et al., 1981) it was found that both antibiotics show the same, square root concentration function on ion permeability. Furthermore measuring the exchange diffusion of cations an equilibrium in the distribution of radioactive ions was not achieved at definite concentrations of either of these substances. The aim of this work is to find an explanation of these unusual properties.

Materials and methods

Human blood from healthy volunteers prevented from clotting by addition of citrate solution and stored at 4 °C not longer than 4 days was used. The blood was equilibrated with the radioactive isotope of the ions to be measured (i.e. ^{137}Cs, ^{86}Rb, ^{42}K) for 1.5 hours at 37 °C. After centrifugation the erythrocytes were washed 3 times with artificial serum solution consisting of 138 mmol/l NaCl, 4.1 mmol/l KCl, 2.5 mmol/l $CaCl_2$ and 1.0 mmol/l $MgCl_2$ (pH 6.8) and resuspended into a given amount of the same solution to achieve the haematocrit value (h) required in the different experiments. (To mark this extracellular ion composition the name "asymmetrical system" is used in the text.) The same treatment was made in tracer exchange experiments, too, but instead of artificial serum solution a solution consisting of 130 mmol/l KCl, 20 mmol/l NaCl, 2.5 mmol/l $CaCl_2$ and 1.0 mmol/l $MgCl_2$ was used for washing and resuspending the erythrocytes (symmetrical system). The suspension was put into a shaking bath at 20 °C and samples were taken out in given intervals. The samples were centrifugated and aliquot parts of the supernatants were measured for radioactivity. For ensuring the exact mixing, the first sample was taken only in 15–17 minutes after resuspension and was considered as control value. Then either gramicidin (in concentration range of 10^{-11} to 10^{-9} mol/l) or primycin (3×10^{-6}–10^{-5} mol/l) was added into the suspension from a stock solution. Gramicidin was solved first in ethanol (10^{-3} mol/l) and before using diluted with methanol to a concentration of 10^{-6}–10^{-7} mol/l, while primycin was stored in 10^{-3} mol/l methanolic solution. The concentration of alcohols never exceeded 1 per cent v/v and they, by themselves, had no effect on the cation permeability.

The transport of cations was characterized by the amount of radioactive ions effluxed and was expressed in the percentage of the total activity of blood (N_t).

Gramicidin was obtained from Sigma Co., primycin Chinoin Pharmaceutical Works (Budapest, Hungary). All salts used were of analytical grade.

Results and discussion

Extracellular primycin increases the cation permeability of erythrocyte membranes both in symmetrical and in asymmetrical systems. Figure 1 shows typical curves for ^{42}K efflux at 10^{-5} mol/l primycin concentration.

Similar results were obtained on gramicidin modified human erythrocytes.

In the following the symmetrical system was used as extracellular medium and the exchange diffusion of cations was measured. The transport values were treated according to the standard procedure of exponential analysis (Kotyk and Janácek, 1970). In Fig. 2 the kinetical data of ^{42}K efflux of primycin (2/a) and gramicidin (2/b) modified erythrocytes are presented in semilogarithmic plots. This representation convinced us that these transport processes could not be described by one exponential function such as it would be expected for simple exchange diffusion. At least two exponential terms were required to fit the experimental results.

Hereinafter the statistical mechanical model is presented to explain the unusual kinetic features of these transport curves. This is an application of Adam's model for the cooperative transitions in biological membranes (Adam, 1969). We started from the qualitative picture got on the basis of the results of our recent ESR experiments with primycin: the antibiotic molecules entering the membrane create structural defects in the neighbouring lipid molecules. Thus, the penetration of a molecule into the place of the membrane already containing an antibiotic is energetically easier then into an unmodified membrane. It has to

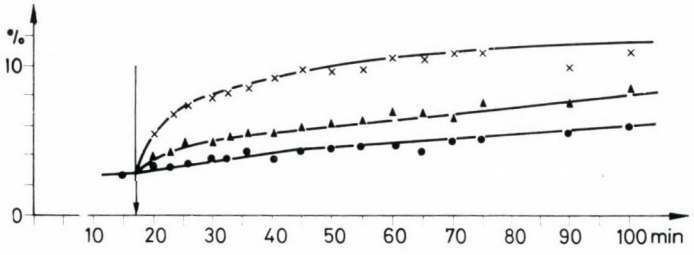

Fig. 1. Time dependence of ^{42}K effiux at 10^{-5} mol/l extracellular primycin concentration. Haematocrit value: 0.4, temperature of incubation: 20 °C. Ordinate gives the amount of radioactive ions transported, expressed as a percentage of the total activity. ● — ● — ● control; ▲ — ▲ — ▲ symmetrical system; × — × — × asymmetrical system. For details see the text. The points in all Figures represent the average of four samples taken from the same blood

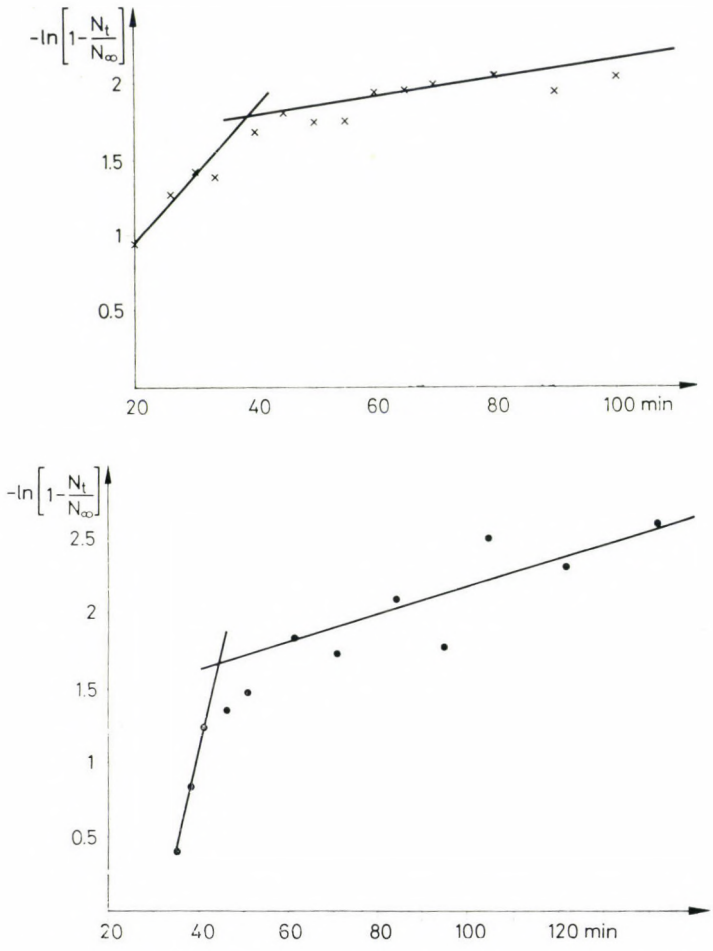

Fig. 2. Time dependence of ^{42}K effluxes in semilogarithmic presentation at 10^{-5} mol/l extracellular primycin (2a) and 10^{-9} mol/l extracellular gramicidin (2b) concentrations. Haematocrit value: 0.4, temperature of incubation: 20 °C. In the ordinate the symbols N_t or N_∞ are the specific activities at the time of sampling or at isotopic equilibrium. Extracellular medium is symmetrical. For details see the text

be emphasized that it does not mean specific affinity for antibiotics, but it means that the binding of a new antibiotic molecule to the neighbourhood of the first one requires less energy than that necessary for the perturbation of the intact lipid matrix. As a consequence aggregations of antibiotic molecules may occure in the membrane which are responsible for ion passing.

In the model the following assumptions are made in order to diminish the number of parameters:

1. there are many but a limited number of binding sites on/in the membrane (N)

2. the number of antibiotic molecules is much higher than N;

3. the rate limiting step of the radioactive ion efflux is the binding of antibiotic to the membrane;

4. the lateral motion of an antibiotic in the membrane is disregarded, the incorporated molecule interacting with its surroundings either stays in place or is released from the membrane.

There are binding sites in the membrane which bind antibiotics from the extracellular space. The number of binding sites neighbouring one site is z (coordination number). When a binding site is occupied by an antibiotic molecule the site is in state 1, when it is empty the site is in state 0. For a particular site the probability of transition from one state to the other depends on the actual states of the next neighbouring binding sites. Some of these sites (a) are in state 1, whereas (z–a) are in state 0. Thus, the probabilities W_{01} and W_{10} of the transition $0 \rightarrow 1$ and $1 \rightarrow 0$, respectively (Adam, 1969) can be expressed as follows:

$$W_{01}(z, a) = k_0' \exp\left[w_{00}(z-a)\,(kT)\right] \cdot \exp\left[w_{01}\, a/kT\right]$$
$$W_{10}(z, a) = k_1' \exp\left[w_{11}\, a/kT\right] \cdot \exp\left[w_{01}(z-a)/(kT)\right]. \qquad (1)$$

Here w_{00}, w_{01}, w_{11} are energies arising from interactions 00, 01 and 11 pairs of nearest neighbours, respectively. k_0' contains the interaction energy between the antibiotic and the membrane (E_i) and is proportional to the amount of the antibiotic per one binding site (c_0/N). Thus:

$$k_0' \sim \exp\left[-E_i/kT\right]\left[\frac{c_0}{N} - g_1(t)\right] g_0(t)\,, \qquad (2)$$

where g_0 is the number of empty binding sites and g_1 is the number of binding sites occupied by antibiotics, k is Boltzmann's constant, T is temperature. Similarly:

$$k_1' \sim \exp\left[-E_0/kT\right], \qquad (3)$$

where E_0 is the energy of an antibiotic molecule for leaving the membrane.

In the approximation used here the partial distribution functions $g_i(z, a)$ for a site in state i ($i = 0, 1$) and its z nearest neighbours (a of which are in state 1) can be written (Volkenstein et al., 1962; Gotlib, 1962)

$$g_i(z, a) = g_{i0}^{(z-a)} \cdot g_{i1}^{a}/g_i^{z-1}; \qquad i = 0, 1 \qquad (4)$$

where g_{00}, g_{01}, g_{11} are the pair distribution functions; g_0 and g_1 are the single distribution functions, the indeces indicating the states. Neglecting the distribution functions of higher orders the amount of aggregates of antibiotic molecules should be taken proportional to g_{11}. In the case of $g_{11} \ll 1$ not every erythrocyte has

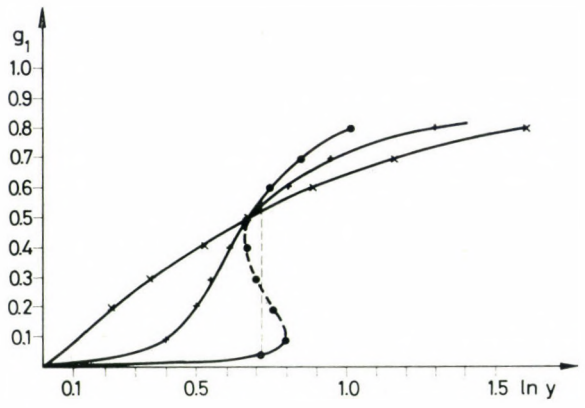

Fig. 3. Plot of equilibrium values of g_1 versus log y. The parameters used in calculations are: $z = 4$, $w_{11} = w_{00}$, q values: ● − ● − ●: 3.49; + − + − +: 2.71; ×−×−×: 2.117. For explanation of symbols see the text. The broken part of the curve represents thermodynamically unstable region

aggregates, in other words some erythrocytes are not modified. So the amount of modified erythrocytes is also proportional to g_{11}. Similar proportionality may exist between the amount of ions effluxed and g_{11} because the pathways created by the aggregates have been assumed to result in a high transport rate for ions. Although this proportionality is correct only for condition $g_{11} \ll 1$, in the followings the same proportionality is applied, as a first approximation, for the more general case ($g_{11} < 1$), and g_{11} as a measure of ions transported will be calculated.

The following obvious relations exist between these partial distribution functions:

$$g_{00} + g_{01} = g_0; \quad g_{01} + g_{11} = g_1; \quad g_0 + g_1 = 1 \tag{5}$$

The Master equations of the system:

$$\frac{\mathrm{d}g_1}{\mathrm{d}t} = \sum_{a=0}^{z} \binom{z}{a} W_{01}(z, a)\, g_0(z, a) - \sum_{a=0}^{z} \binom{z}{a} W_{10}(z, a)\, g_1(z, a)$$

$$\frac{\mathrm{d}g_{01}}{\mathrm{d}t} = g_{00} \sum_{a=0}^{z-1} \binom{z-1}{a} W_{01}(z, a) g_0(z-1, a) - g_{01} \sum_{a=0}^{z-1} \binom{z-1}{a} W_{01}(z, a+1) \cdot$$

$$\cdot\, g_0(z-1, a) + g_{11} \sum_{a=0}^{z-1} \binom{z-1}{a} W_{10}(z, a+1)\, g_1(z-1, a) -$$

$$- g_{10} \sum_{a=0}^{z-1} \binom{z-1}{a} W_{10}(z, a)\, g_1(z-1, a)\,.$$

Solving these equations by method of Runge–Kutta, the time course of g_{11} ($g_{11} = g_1 - g_{01}$) can be evaluated.

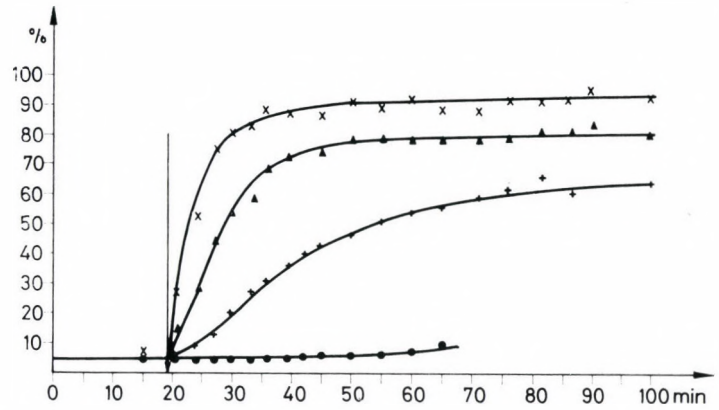

Fig. 4. Time dependence of ^{42}K effluxes at different gramicidin concentrations. Haematocrit value: 0.06, temperature of incubation: 20 °C. The ordinate gives the amount of radioactive ions transported, expressed as a percentage of the total activity. Extracellular medium: symmetrical. For details see the text. Extracellular gramicidin concentrations: ● — ● — ●: control, + — + — +: $2.7 \cdot 10^{-10}$ mol/l, ▲ — ▲ — ▲: $1.35 \cdot 10^{-9}$ mol/l, × — × — ×: $2.7 \cdot 10^{-9}$ mol/l

The results of solutions of the Master equations for equilibrium cases are given in Fig. 3. In the figure we plotted g_1 values versus ln $y(y = \exp[-(E_i - E_0)/kT]c_0/N)$, where c_0/N is the number of antibiotics per one binding site and c_0 and N are taken infinite; q values characterize the degree of cooperativity ($q = \exp[-w/2\,kT]$, where $w = w_{00} + w_{11} - 2\,w_{01}$). As it can be seen, the larger the value of g_1 the more energy is required for antibiotic's entering the membrane. At low q values the number of penetrating antibiotic molecules increases gradually with increasing antibiotic concentration, while in the case of high q values a critical antibiotic concentration exists above which a sudden increase occurs in the number of antibiotics bound in the membrane. Our experimental results are in good agreement with this model. As it is shown in Fig. 4 the equilibrium ion distribution between the intra- and extracellular compartments of gramicidin modified erythrocytes was achieved by increasing the antibiotic concentration up to 2.7×10^{-9} mol/l at a haematocrit value of 0.06. Below this concentration, however, the kinetic curves prove the existence of not "perfectly" modified erythrocytes, too. The exponential representation of these kinetic data clearly show the presence of unmodified cells giving us a direct proof of the validity of the model (Fig. 5). The slopes of the second exponents are the same at every concentration of both antibiotics and correspond to the control transport rate (0.002–0.003 min^{-1}). In the case of primycin the critical concentration could not be reached because this antibiotic can be applied only in a very narrow concentration range without cell damage (10^{-6}–$5 \cdot 10^{-5}$ mol/l).

When considering the g_{11} value of the model and its correlation with the ion flux, first the time course of g_{11} values is shown at arbitrary antibiotic concentrations in Fig. 6.

For comparison, in Fig. 4 and Fig. 7 our experimental results are given for gramicidin and primycin, respectively. Good qualitative agreement can be seen

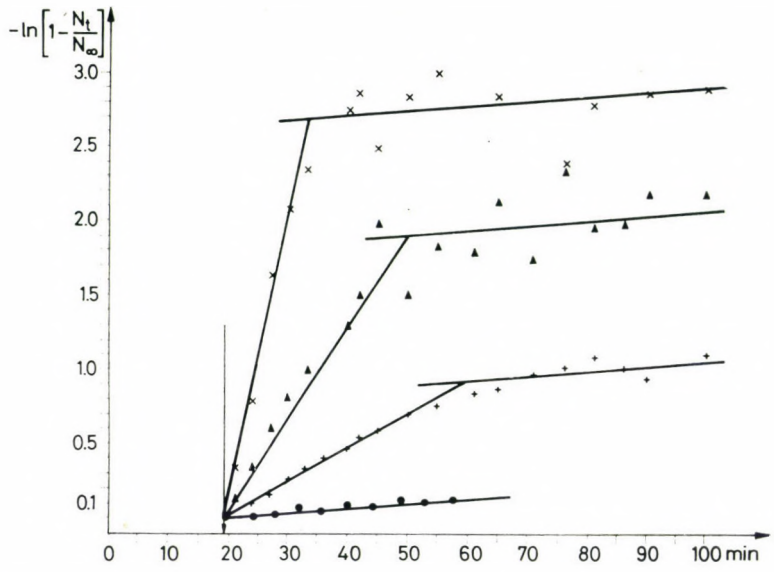

Fig. 5. Semilogarithmic representation of data shown in Fig. 4. In the ordinate the symbols: N_t or N_∞ are the specific activities at the time of sampling or at isotopic equilibrium

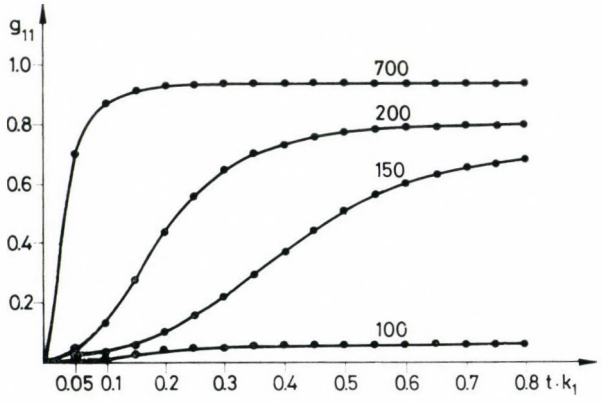

Fig. 6. Plots of g_{11} values versus $t \cdot k_1$. Here t is time interval, k is constant containing the interaction energies. The parameters used: $q = 3.49$, $z = 4$, $y' = \exp -(E_i - E_0)/kT = 0.02$. [If $c_0/N\, g_1(t)$ than $y = y'\, c_0/N$.] $w_{11} = w_{00}$

between the theoretical and experimental curves. This finding allows us to accept this model which explains the molecular mechanism of the action of these antibiotics on red blood cells. Moreover, it also proves the similarity in the effects of the two compounds on red blood cell membrane. However, there is remarkable quantitative difference between the effects of these substances: the primycin

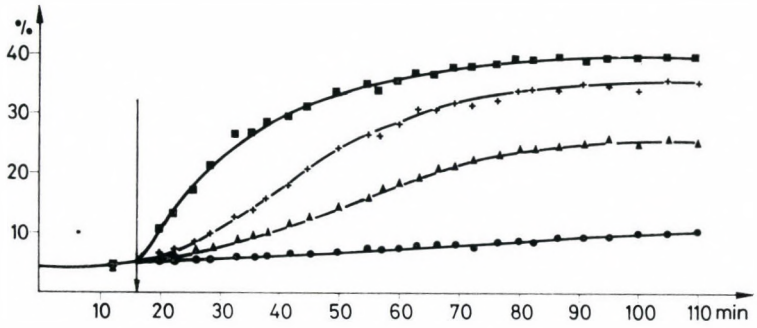

Fig. 7. Time dependence of ^{42}K effluxes at different primycin concentrations. Haematocrit value: 0.06, temperature of incubation: 20 °C. The ordinate gives the amount of radioactive ions transported expressed as a percentage of the total activity. Extracellular medium: symmetrical. For details see the text. ● — ● — ●: control; ▲ — ▲ — ▲: $3.15 \cdot 10^{-6}$ mol/l; + — + — +: $6.3 \cdot 10^{-6}$ mol/l; ■ — ■ — ■: $1.26 \cdot 10^{-5}$ mol/l

induced efflux values are far from the equilibrium distribution of radioactive ions although primycin was applied in much higher concentrations than gramicidin. To understand this difference the partition coefficient of these compounds between the membrane and media was estimated and it was found 1 to 10 and 10^4 for primycin and gramicidin, respectively. Taking into account these results and the rather narrow concentration range in which primycin can be applied without cell damage it is obvious that the extracellular primycin concentration was always below the critical concentration.

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Estimation of Zinc and other Trace Elements in the Nails of Hungarian Adult Twin Pairs by Neutron Activation Analysis

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Nail samples of 157 adult Hungarian twin pairs were examined for different trace elements by neutron activation analysis. Comparing the within-pair-concordance for zinc contents of the twins of different zygosity, a much higher concordance in monozygotes than in dizygotes was observed. The authors suggest the idea that the zinc content in the human organism, at least in some organs, may be genetically controlled.

Introduction

Trace elements in the human organism are not major components of the body build, and their role has not yet been completely elucidated, but by the application of modern micro-analytical technique they have become exactly measureable. Nevertheless, physiological research made doubtless that they are vividly functioning in various metabolic processes, growth, enzymatic activities, wound healing, etc.

The normal levels of the various essential trace elements are definitely limited to certain ranges. Their concentration values do not normally deviate more than ± 20 per cent. Since the normal values (e.g. in the blood serum) are somewhere in the order of 10^{-6} and 10^{-9} g/ml (*Nucl. Act. Techn. of the Life Sciences*, 1976), a highly sensitive and effective procedure for their analysis has been requested. For this reason, in different works the neutron activation analysis and atom-absorption-spectrometry was applied.

Our basic concept was that the concentration of the trace elements in human organism must be genetically influenced (Taylor et al., 1973; Siegers et al., 1977), though this content may obviously be modified by environmental factors. In order to penetrate into the problem, 157 pairs of adult Hungarian twin pairs were examined.

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Materials and methods

Examinations took place at the Anthropologic Institute of the Eötvös Loránd University, Budapest, Hungary. Dizygotic twins with members of dissimilar sexes were excluded. The zygosity (monozygotic, *MZ* or dizygotic, *DZ*) of the pairs was mainly based on blood group determinations (Rex-Kiss et al., 1974), but further diagnostic means, anthropometry, dermatoglyphic measurements, phenylthiocarbamide taste sensitivity, etc. were also taken into account. 64 DZ and 93 MZ twins were found. Fingernail clippings were obtained from each person from the IVth finger of the left hand for trace element measurements and the data of MZ and DZ twins were compared. Nail samples were cut with nickel-plated scissors. The dirt of nails was cleaned off with a nickel knife, all the samples repeatedly washed by bidistilled water and finally cleaned by using the technique of *Jervis* (1967) and a methanol-aceton solution. After drying and weighing, the samples were put into quartz ampoulles, then the ampoulles were sealed and submitted to a long-time irradiation in a Merlin reactor of the Nuclear Research Center of Jülich, West Germany. (Neutron flux = $5 \cdot 10^{13}$ n/cm$^2 \cdot$ s.) A simultaneous and non-destructive method for the estimation of trace elements was by neutron activation analysis obtained.

After setting disturbing radio-isotopes, as e.g. Na-24, aside, the samples were removed into not-irradiated test glasses and gamma spectra were obtained using a Ge (Li) detector (100 cm^3) joined up with a multichannel analyser (2048 channels). Iron, as a nearly 1/v-type isotope was used as a flux monitor and comparator element for the other 1/v-type isotopes, as for zinc, cobalt, etc. Data were obtained with the help of an element library by a computer evaluation.

Results and discussion

Nail clippings were analysed for 14 elements: Co, Fe, Se, Sb, Rb, Hg, Zn Cs, Ag, Sc, Ni, Cr, K and Mn. Since our method was not specifically elaborated for any one of the elements studied, and it was worked out to get the maximum possible information from one or two measurements, a good number of results represent only an upper level of concentration compared to which the real concentration of the element was lower. In a significant proportion of the material there were only 7 elements for which concrete values were obtained. When the intrapair variability of the average levels of various elements was compared in MZ and DZ twins, only in zinc values was a remarkable concordance found.

In Fig. 1 the mean of the differences for 7 trace elements within MZ twins compared to DZ twins is demonstrated. Since each sample offered zinc values that could be estimated and just for this element the concordance was convincing, the zinc data were analysed further.

In Fig. 2 the concentration frequency of all zinc values is shown, irrespective of the fact that they originated from different types of twins (though it was clear

to us that twin data cannot be treated in the same way as can data from a normal population without any restriction). The mean value and the Gaussian distribution corresponded well with literature data, despite that these data have been obtained from different geographical areas (Kozuka, 1972; Liebscher et al., 1968). The mean level of zinc in the nails was found $1.49 \cdot 10^{-4}$ g/g $\pm$ $0.92 \cdot 10^{-4}$ g/g. It is interesting that the nail samples taken from 18 cadavers by Liebscher and Hamilton Smith (1968) in Glasgow showed very similar results to ours.

The nail zinc content distributions of 128 male and 128 female subjects – judging from the material – show the same phenomena as found by Kozuka (1972) in Japanese hair samples, namely that in the case of females the trace element contents were distributed in a wider range (Fig. 3).

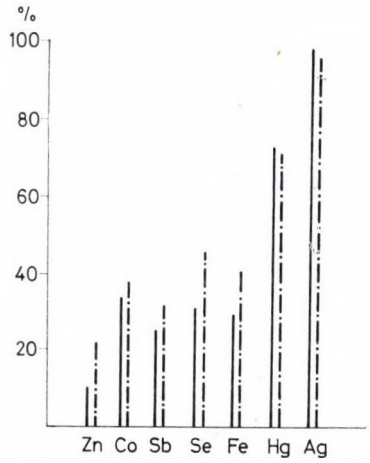

Fig. 1. Mean values of the differences within MZ and DZ twins for 7 trace elements in human nail samples (full lines: MZ, dotted lines: DZ)

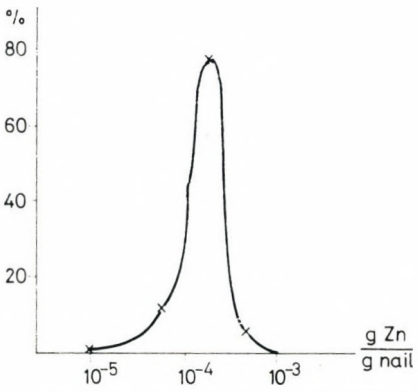

Fig. 2. Concentration frequency of all zinc values

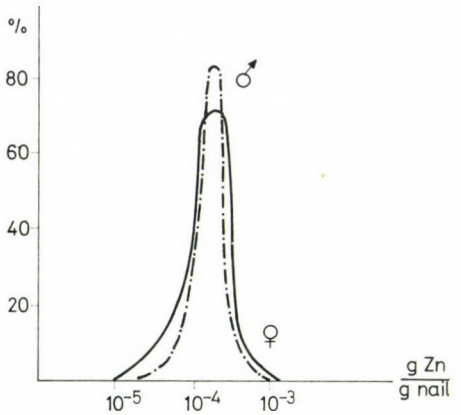

Fig. 3. Nail zinc content distribution in female and male samples (full line: female, dotted line: male)

No correlation was found among different ages, sexes, professions and habitations of the twin pairs (living together or apart).

Nevertheless, there may be a slight correlation between twin pair data if both members are living together and taking the same foods, but different professions, hobbies, drug intake, etc. probably have a stronger opposite effect.

In order to compare the percentual number of zinc content deviations within MZ and DZ pairs, the following formula was used:

$$\Delta X\,(\%) = \frac{100\,|X_a - X_b|}{\frac{X_a + X_b}{2}}$$

where ΔX is the zinc content deviation within one pair and X_a and X_b are the zinc concentrations (in g Zn/g nail) from the members of the twin pair. Figure 4 represents the frequency distribution of ΔX showing that the within-pair-concordance for zinc contents is much higher in MZ than in DZ twins. [$\chi^2_{DZ}/\chi^2_{MZ} = 2.31$]

For the 75 per cent of MZ pairs, the mean of the ΔX, i.e. $\overline{\Delta X} = 7.6\%$ (SD 5.5),
for all the MZ pairs

$$\overline{\Delta X} = 15.9\%\ \text{(SD 16.9)},$$

versus in the DZ pairs

$$\overline{\Delta X} = 22.8\%\ \text{(SD 21.4)}.$$

In the 25 per cent of the MZ data the rather high deviation found may be attributed to different environmental factors. As an example, in many cases the members of the pairs have quite different professions: taking the case of one set

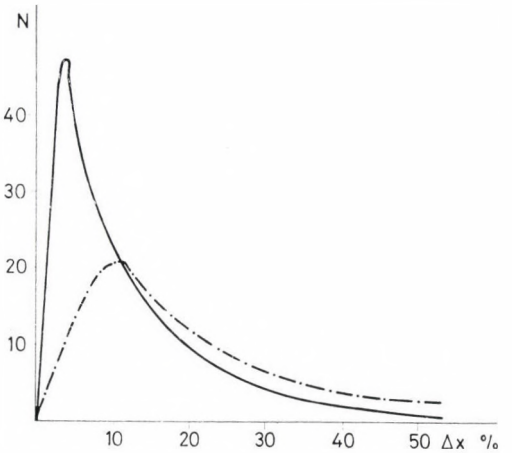

Fig. 4. Frequency distribution of ΔX values (full line: MZ, dotted line: DZ) for zinc levels

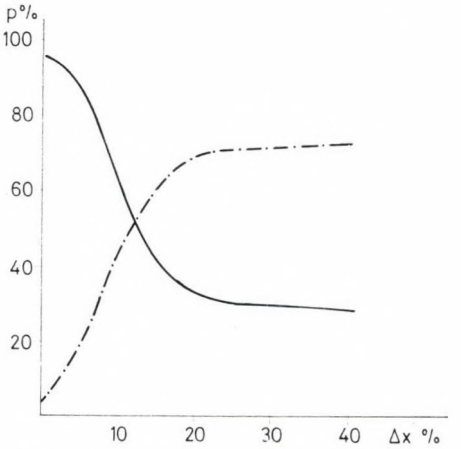

Fig. 5. Probability for being MZ or DZ on the basis of the within-pair deviations

of our twins, one worked as a nurse in a children's hospital and had much to do with zinc-containing ointments whereas the other was a clerk (accompanied by much higher zinc levels in the nail sample of the first one).

Some consequences concerning zygosity can be drawn in certain pairs from concordance or discordance in nail zinc content. Figure 5 indicates the probability for being mono- or dizygotic on the basis of their within-pair zinc deviation. For instance, if the deviation is 5 per cent, there is a 83 per cent probability for being MZ, while in the case of a deviation of 20 per cent, the probability is 35 per cent. (The estimation was constructed on the basis of data of 58 DZ and 82 MZ twins.)

The fundamental aim of our investigation was not to elucidate the significance of zinc in biological processes, rather to show that its level in the healthy human organism may be influenced by genetic factors. If more elements and larger populations were examined, some other elements might show a similar concordance. The concept of human polymorphism and "genetic individuality" have been discussed in many publications (Williams, 1956, 1967; Childs, der Kaloustian, 1968; Forrai, Bánkövi, 1975 etc.). In this work, we tried to approach the physical/biochemical make-up of the body build in a new way. Furthermore, trace element (zinc) contents of different human organs, if compared in MZ and DZ twins, may be acceptable as a new diagnostic possibility for the determination of zygosity.

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Temperature Dependence of ATP Release from "Caged" ATP

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The ATP release from "caged" ATP by laser flash was determined at different temperatures and pH values. We measured the time resolved absorbance changes of the coloured intermediate, which is involved in the photolytic process. The linerity of the Arrhenius curves suppose only one process and the calculated activation enthalpies (arround 55 kJ/mole) did not change markedly at the pH values of 6.0, 7.0 and 8.0.

One of the most difficult problems in molecular studies of biological systems is to introduce the reactants to the reaction sites in a time shorter than the characteristic time of the reaction under investigation. A new approach of time-resolved studies for ATP-requiring biological systems is under development. A derivative of ATP, P^3-1-(2-nitro) phenylethyladenosine 5'-triphosphate, called "caged" ATP, has been synthetized (Kaplan et al., 1978).

This ATP derivative is inactive in biological systems but may be activated by UV irradiation which releases the ATP from its "cage". The mechanism of conversion of caged ATP to ATP was given by the spectroscopic analysis of McGray et al. (1980). They found that the amount of ATP produced is linearly related to the laser energy and that an intermediate is involved in the photolytic process: [Caged ATP (I) $\xrightarrow{h\nu}$ a coloured aci-nitro compound (II) + proton; compound II $\rightarrow$ 2-nitrosoacetophenone (III) + ATP]. This intermediate (II) has an absorption different both from ATP and caged ATP. Therefore, it is possible to study the decay of compound II with spectroscopic techniques. This decay is the rate limiting step in ATP formation and has a rate constant of 2.2×10^9 (H^+) sec^{-1} at 22 °C.

In the work described here, the temperature and pH dependence of the decay rate of compound II was measured after laser flash photolysis of caged ATP and the activation enthalpy of this process was determined.

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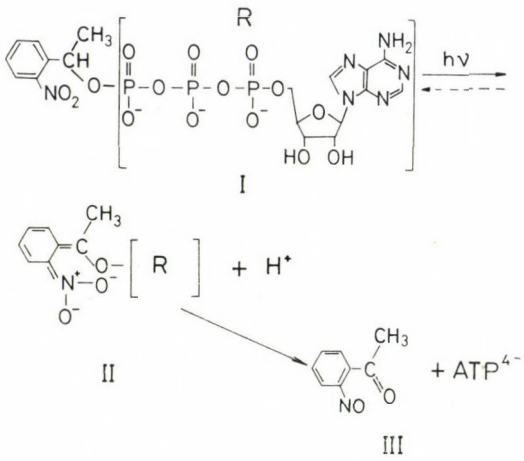


Fig. 1. The chemical steps of the conversion of caged ATP to ATP

Materials and methods

Caged ATP was prepared by the method of Ludwig (1982). This method is a modification of that of Kaplan et al. (1978) and yields caged ATP with the same spectroscopic and chromatographic properties. 10 mM caged ATP was dissolved in 10 mM Tris buffer or distilled water at pH 6.0, 7.0, 8.0.

Measurements were performed in a UV cell with an optical pathlength of 0.5 cm. A Peltier thermocouple was used to control the temperature. A nitrogen laser produced the actinic light (λ_{max} = 340 nm, half time ~ 5 ns, energy ~ 0.5 mJ) and a stabilized Xenon lamp provided the continuous measuring light. Two monochromators (Oriel Model 7240) were used to select the wavelength absorbed by intermediate II (λ = 400 nm). A transient recorder (product of the Central Research Institute for Physics, Hungary; smallest conversion time 0.1 μs, conversion range 256 quanta/channel, 1024 channels) digitalized the changes of light intensity, which were detected by a photomultiplier (EMI 9526/B). A sophisticated on-line measuring and data processing system (CAMAC) was used to control the measurement, to average and store the signals and to compute the absorption changes (Fig. 2).

Results and discussion

Two series of measurements of absorption changes were performed at different pH values, namely at pH 6, 7 and 8, one series in buffered, the other in non-buffered solutions. The pH levels of the solutions were adjusted before the experiments and pH changes which occurred during the measurements were not monitored. In all cases the intensity changes were exponential and the ampli-

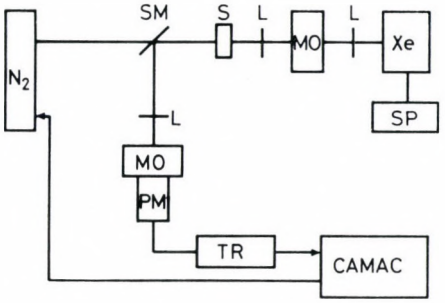


Fig. 2. Sheme of the measuring system. N_2: nitrogen laser; SM: semitransparent mirror; S: sample; MO: monochromator; Xe: Xenon lamp; SP: stabilized power supply; PM: photomultiplier; TR: transient recorder; L: lens

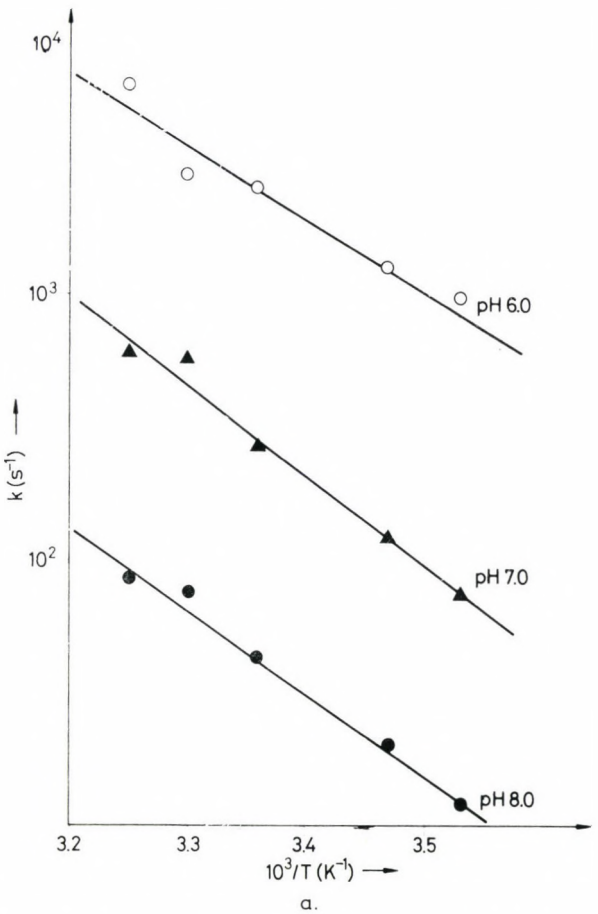
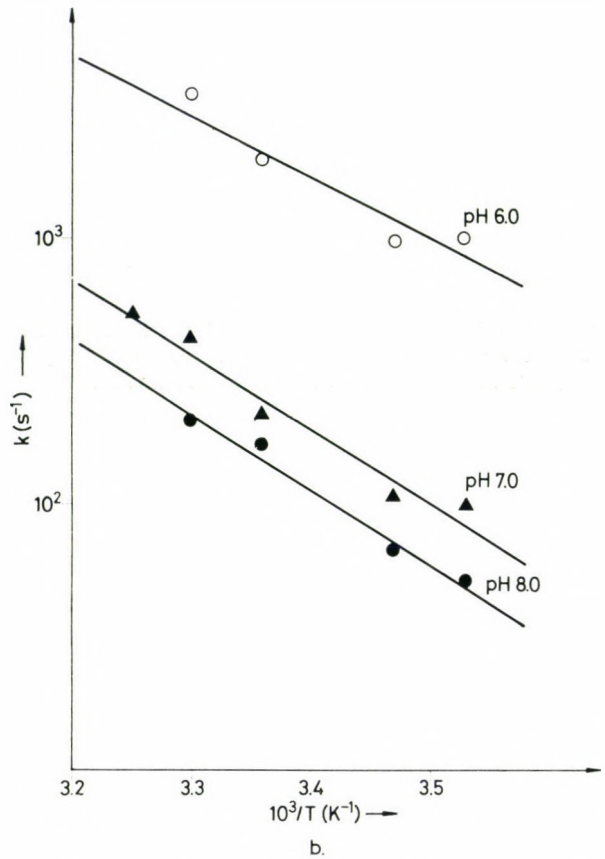


Fig. 3. Rate constants of the photolysis of caged ATP at different pH. a) Tris (amino) buffer;



b) distilled water. The measured data (prints) were fitted by the Arrhenius equation (eq. 1)

tudes were constant. The rate constants are displayed in Figs 3a and b. It can be seen that the transition rates follow the Arrhenius equation:

$$K(\mathrm{T}) = A\, e^{-\Delta H/RT}$$

where ΔH, the activation enthalpy and A, the frequency factor, are taken to be temperature independent. Increasing the temperature by 10 °C increases the rate by about a factor of two. As expected, the linearity of these curves shows the appearance of only one process. Similar curves were obtained in water (Fig. 3b) where the lack of buffering capacity resulted in an upward shift of the curve measured at pH 8 toward the pH 7 curve, compared with curves in buffer (Fig. 3a). This is in agreement with the fact that the rate of ATP release is pH dependent (see Fig. 3) and proton release is involved in the process. The calculated activation enthalpy shows no discernible pH dependence (Table 1). It is possible to estimate

Table 1

Activation enthalpy (in kJ/mol K) *of the decay process of the intermediate at different pH values*

	In 10 mM Tris (amino)	In water
pH 6	52.1±7.1	45.1±9.8
pH 7	65.6±5.6	53.6±6.5
pH 8	59.0±2.1	53.8±5.0

the amount of ATP produced by comparing Figures 3a and b. Approximately 1 out of every 10^5 molecules of caged ATP is converted to free ATP.

This 2-nitrobenzyl derivative of ATP is stable in water, it was found to be neither a substrate nor an inhibitor of purified Na, K-ATPase and it could be incorporated into membranes (Kaplan et al., 1978; McGray et al., 1980). These characteristics make caged ATP a very promising compound in time-resolved studies of both intact and soluble biological systems.

The authors are very much indebted to Dr. J. Czégé for helpful suggestions in the experiments. Thanks are due to Dr. B. Sarkadi for discussions and to Dr. J. Ludwig for synthetizing the caged ATP.

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The Effect of Adenosine Diphosphate on the Interaction of Actin-Myosin-Adenosine Triphosphate

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It was shown in experiments made on natural actomyosin of rabbit skeletal muscle that ADP possesses direct and indirect effects on superprecipitation. The presence of ADP increases the turbidity of the actomyosin suspension. An optimal ATP/ADP ratio may help the development and existence of a PC conformer of actomyosin corresponding to a myosin-ADP-actin-myosin-ATP complex. In this complex the extent of actin-activation is controlled by the cooperativity between the two myosin heads which, on the other side, may regulate in vivo the optimal transformation of the chemical energy of ATP for mechanical work.

Introduction

In agreement with some recent observations (Kakol et al., 1980; Knyrim, 1981) we found that superprecipitation of natural actomyosin (NAM) of rabbit skeletal muscle develops in two phases in a reaction mixture containing KCl in near-physiological concentration ($\sim$ 0.140 M; 6.26). We have proved that the steps of superprecipitation are synchronous with step-like changes in the concentration of ATP and ADP (Csabina et al., 1982).

Numerous data have been found recently, showing that the ADP complex of ATPases may influence the kinetics of the enzyme reactions in different biological systems, that is, it may exert regulatory functions in definite biological processes. Among these we may mention its role either in the control of mitochondrial respiratory chain (Chance, Williams, 1955; Jacobus et al., 1982; Joachim et al., 1982), or in the metabolic reactions of the cellular energy transfer (Askari, Huang, 1982) or in the function of myosin ATPase (Chock, 1981a; Chock, 1981b; Nanninga, 1962; Shriver, Sykes, 1981).

On the basis of the comparison of our results and these data we assumed that ADP constituting a complex with the myosin ATPase may play a basic role in the synchronization of the superprecipitation and in the changes of nucleotide concentration. This complex may play a role "in vivo" in the control of the cyclic

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movement of the cross-bridges, in the cooperative function of the dimer myosin heads and, consequently, in the determination of the parameters of the muscle contraction, too. We, therefore, extended our study of superprecipitation to the effect of ADP.

Materials and methods

Natural actomyosin was prepared from rabbit skeletal muscle according to Ebashi, 1961. The reaction mixture was composed in a photometer cuvette in 3 ml final volume, and changes in its turbidity were registered at 660 nm by Spectronom 195 spectrophotometer at 25 °C. During superprecipitation the reaction mixture was constantly stirred (Ebashi, 1961; Szöőr, Boross, 1979). The control reaction mixture contained 1 g/dm^3 actomyosin, 0.14 M KCl, 0.001 M $MgCl_2$, 0.0001 M $CaCl_2$, 0.02 M TRIS-HCl; pH = 7.0 and 0.001 M ATP. Variations of the composition in the reaction mixture are indicated in the text. The development of the superprecipitation was started by the addition of the nucleotides. The concentration of the protein was determined by biuret reaction calibrated by micro-Kjeldahl method. The chemicals used were of reagent grade.

Results

In the first series of our experiments, the reaction was started by a nucleotide mixture in which the concentration of ATP was constant (0.001 M) and the concentration of ADP was changed (0.0; 0.001 and 0.003 M). Results shown in Fig. 1 indicate that the presence of ADP, in dependence of its concentration,

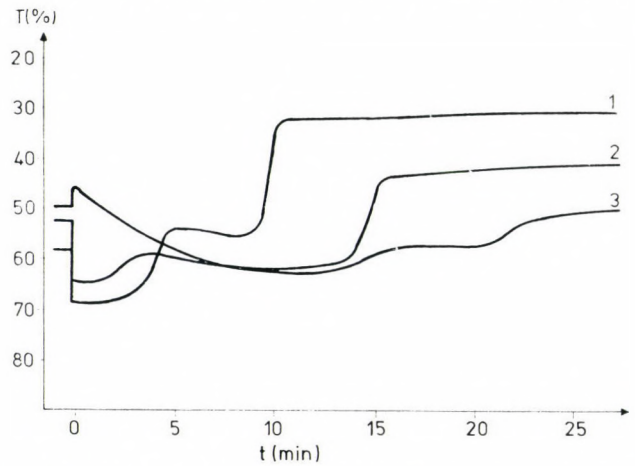

Fig. 1. The effect of ADP on the superprecipitation of actomyosin. The ATP concentration was held constant (0.001 M), and the ADP concentration was varied (0.00; 0.001 and 0.003 M in curves 1, 2 and 3, respectively

shortens or eliminates the clearing of the suspension and causes a delay in the full development of the superprecipitation. As a result of these two effects the duration of the first phase is lengthened. As a consequence of the increase in the total nucleotide concentration, the turbidity of the system decreased. The analysis of the step-like character of the superprecipitation was published elsewhere (Csabina et al., 1980; Csabina et al., 1982; Szöőr et al., 1982).

In the second series of our experiments the total nucleotide concentration was held constant (0.001 M), while the ATP/ADP ratio was changed (Fig. 2 B).

Variations in the curves of column A in Fig. 2 can only be explained by the changes in the initial ATP concentrations (control experiments). A comparison between the pair curves in column A and B directly shows the effect of ADP

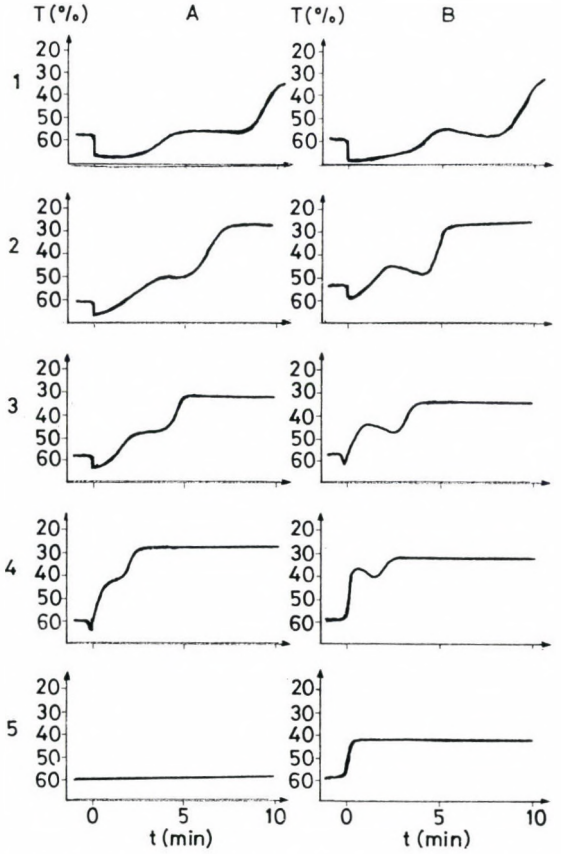

Fig. 2. The effect of the variation of ATP/ADP ratio on the superprecipitation of actomyosin at constant (0.001 M) nucleotide concentration. The amount of ATP is the same in the pair curves of column A and B. Curves in column A are registered without that of column B with proper amount of ADP. The percent of ADP was 0, 25, 50, 75, and 100 in the curves of column B read from above

on the development of superprecipitation. A decrease in the ATP concentration shortens both the duration of the first phase and the time necessary for the full development of superprecipitation (column A). In the presence of ADP the clearing phase decreases or disappears and the amplitude of the first phase increases. In this case the turbidity of the system increases, in spite of the fact that the total nucleotide concentration remains constant.

Discussion

For the interpretation of our results a contraction model was constructed (Kónya et al., 1982) which is outlined in Figs 3 and 4 and Table 1.

In Fig. 3 a typical step-like superprecipitation curve is shown. To the sections tangential straight lines are drawn. Their steepness and duration serve as basis for quantitative evaluation of the parameters of the curve. Horizontal sections of the superprecipitation curve (i.e. the zone in which the turbidity of the mixture is practically unchanged) were regarded characteristic for a definite conformation of the actomyosin, which had specific optical behavior as well. Therefore, tangentials of these sections were given a rational name describing a supposed state of the muscle contraction and assumed to be appropriate for the indicated period of superprecipitation.

The meaning of R, RL, PC and RN letters are explained in Table 1, their correlation with the superprecipitation curves and the model are shown in Figs 3

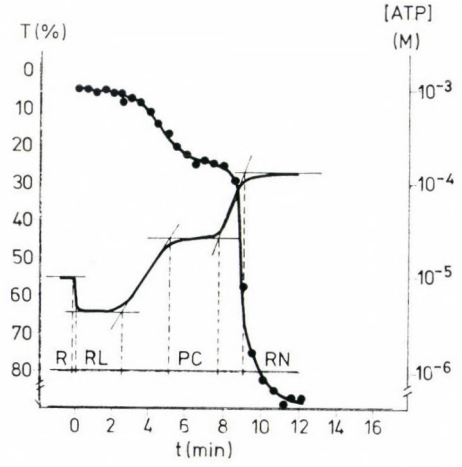

Fig. 3. Sections of step-like superprecipitation curves: R = rigor conformer without nucleotides; RL = conformer corresponding to relaxation; PC = conformer corresponding to physiological contraction; RN = rigor conformer containing nucleotide (ADP). (The curve showing the results of ATP determination using bioluminescent technique was published previously [7]. With permission of the Publisher)

Table 1

Explanation of the correlation between the sign, name, and composition of actomyosin conformers used to describe sections of the step-like superprecipitation of actomyosin

Sign	Name	Composition	Description
R	Rigor complex without nucleotide	M-A | M-A	Both heads of the myosin dimer are bound to actin but neither of them binds nucleotide
RL	Relaxation	ATP-M A | ATP-M A	ATP is bound to both heads of the myosin dimer, which are otherwise dissociated from actin
PC	Physiological contraction	ATP-M A | ADP-M-A	One head of the myosin dimer is bound to ADP and actin while the other is dissociated from actin and bound to ATP
RN	Rigor complex containing nucleotide	ADP-M-A | ADP-M-A	Both myosin heads are bound to actin and ADP

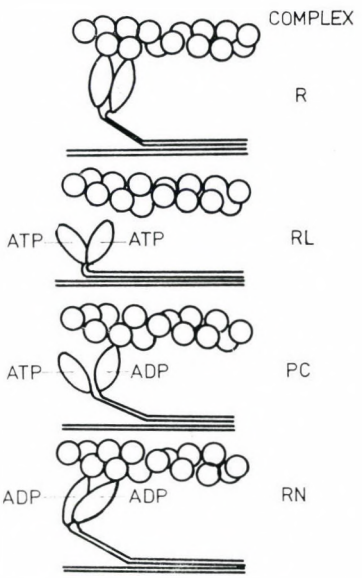

Fig. 4. Model of the molecular composition of actin-myosin-nucleotide complexes. The different conformers are supposed to appear during the step-like superprecipitation of actomyosin

and 4. The transition periods in the curve between these horizontal sections are also characterized by tangentials drawn to the inflection points of the curve. These are assumed to describe the speed of transformation of actomyosin between two conformational states.

On the basis of our model, the phases of superprecipitation as well as the conformational states of actomyosin correlated with the types of interactions between actin and myosin. These interactions differ with the sort of nucleotide bound to the myosin heads. This is confirmed by the changes in ATP concentration during the development of superprecipitation (Fig. 3). During the RP phase the concentration of ATP is relatively high and decreases slowly. In phase PC the concentration of ATP seems to be stabilized transiently, and phase RN develops parallel with the disappearance of ATP.

Actomyosin conformers corresponding to the different phases of the superprecipitation are indicated in Fig. 4 and Table 1. The model depicted in Fig. 4 was constructed on the basis of the results described before, using the contraction model of Schaub (Schaub, Watterson, 1981). According to morphological observations (Harrington, 1979) the structure of myosin has a multidimer structure, similar to the thick filament, while the actin is polimerized (F-actin). On the basis of this model, different conformers of actomyosin are successively transformed from one to another during the development of superprecipitation.

Before starting superprecipitation by adding nucleotides to the actomyosin suspension, actomyosin composes a rigor complex (R). The heads of myosin dimer are bound to actin by hydrophobic interactions and do not contain nucleotides.

Addition of ATP to the suspension dissociates myosin from actin (Weber, 1956) and depending on the ATP concentration, an RL phase appears on the superprecipitation curve. As a consequence of the decrease in ATP and increase in ADP concentrations, PC conformer develops, corresponding to the first phase of superprecipitation at optimal ATP/ADP ratio. Later on, following an abrupt reduction of ATP concentration, the RN conformer appears, which corresponds to the full development, or second phase, of superprecipitation. In RN conformer ADP is bound to myosin heads, which strengthen the binding between actin and myosin. This ADP complex would give the difference in composition, structure and turbidity between R and RN conformer (curve 1 in Fig. 1, column A in Fig. 2, and see Fig. 3, too).

If ADP is present together with ATP in the nucleotide mixture used for the initiation of superprecipitation, the conditions necessary for the constitution of PC conformer are insured. The sooner the optimal ATP/ADP ratio evolves, the faster the first phase appears on the superprecipitation curve (curve 2 and 3 in Fig. 1, column B in Fig. 2).

The parameters of the step-like superprecipitation curve are depending, beyond an optimal ATP/ADP ratio, on the total nucleotide concentration (compare curves 2 and 3 in Fig. 1 with curves 3 and 4 in column B, Fig. 2). This phenomenon can be explained by the fact that at higher nucleotide concentrations

an optimal ATP/ADP ratio can be maintained longer as the consequence of ATP regeneration (Csabina et al., 1982). In this case a decrease in the turbidity can be explained by the high ATP concentration within the limit of optimal ATP/ADP ratio and, as a consequence, RL conformers of actomyosin are probably simultaneously present in the suspension.

On the basis of our model, which is in good agreement with the scheme of Lymn and Taylor 1970, superprecipitation can be satisfactorily understood as the contraction model of the muscle and it contributes to the completion of previous works (Eisenberg, Hill, 1978; Hill et al., 1981; Inoue, Tonomura, 1980; Nonomura, Ebashi, 1974; Schaub, Watterson, 1981).

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BOOK REVIEWS

Hormone Receptors. In: Horizons in Biochemistry and Biophysics Vol. 6. (Edited by L. D. Kohn) John Wiley and Sons, Chichester–New York–Brisbane–Toronto–Singapore, 1982 pp. 392.

This volume gives a presentation of recent advances in the field of hormone receptors. It is only membrane receptors that are dealt with, i.e. receptors for polypeptide hormones, toxins and interferon. The treatise is deliberately not comprehensive and the number of references is not overwhelming. It is restricted to the most important original papers and several recent reviews. The book is easy to read, since it is intended to give an introduction to this area to advanced learners of biochemistry, biophysics or pharmacology as well as to put forward new ideas to researchers who are specialized in other fields. In keeping with this aim, the articles do not give an exhaustive or detailed description of each hormone receptor, but rather use informations concerning individual receptors to illustrate problems and approaches in the whole area.

In the first chapter, thyrotropin receptor is described in some detail in order to define criteria by which a binding principle may be raised to the status of receptor. Thyrotropin receptor is defined and redefined in the light of emerging experimental facts. The complexity of coupling ligand recognition to hormone effect is also explored. It is shown how the questions and answers can be generalized to receptors for other hormones, toxins and other ligands recognized by the cell, e.g. interferon. This article serves also to call the readers' attention to topics of other chapters in the book.

These latter range from purely theoretical approaches, such as the one dealing with quantitative mechanistic models of hormone binding and elicited response, to discussions of the use of various experimental techniques, such as monoclonal antibodies in receptor research.

Some articles discuss the hormone recognition problem in detail. Separate chapters are devoted to the role of lipids as receptor components and to carbohydrates as recognition determinants. The questions of receptor synthesis, insertion into lipid bilayer and movement through different membrane compartments are raised next.

The ways of receptor signalling to the cell are also addressed, of course. However, the well recognized second messenger system, adenylate cyclase and cAMP, is not discussed in detail, since that is already a text book issue. Four out of the fifteen chapters of the book discuss either the regulation of Ca^{2+} ion transport through plasma membranes by hormones and receptors or the interaction of this system with cyclic AMP, showing that the editor paid particular attention to Ca^{2+} ion signalling.

Finally, three chapters deal with interferons. One of them describes achievements in their purification and characterization, the other gives a survey over some results with interferon inducers and the third reviews our current understanding of the molecular processes which could account for the anti-viral action of interferon.

With this content and style the book, I think, will well serve its aims, it is a good reference to follow and review the data in all receptor fields as they emerge in the next several years. P. ARÁNYI

Acta Biochimica et Biophysica Academiae Scientiarum Hungaricae 19, 1984
Akadémiai Kiadó, Budapest

Neuroreceptors. Proceedings of a Symposium held in West-Berlin September 28–29, 1981, Edited by F. Hucho. W. de Gruyter, Berlin, New York 1982, pp. 359.

The book presents contributions to a meeting held in West-Berlin, 1981, whose participants were from research institutes of the GFR. Reviews and original data were presented on neural receptors that have been in the focus of interest.

The articles are grouped in seven chapters according to the receptors that are dealt with, namely, diazepam receptor, nerve growth factor receptor, dopamine receptor, beta-adrenergic receptor, glycine and tetrahydrocannabinol receptor, central and muscarinic acetylcholine receptor and nicotinic acetylcholine receptor. This last chapter contains by far the most contributions, because, in the opinion of the editor, it is the most interesting and most important molecule of its kind and, besides, its biochemical investigation is, for technical reasons, fairly advanced. The issues discussed in relation to this receptor comprise embryological development of the electric organ of *Torpedo marmorata*, uses of various experimental techniques in acetylcholine receptor research, such as flux amplitude analysis, percoll gradient centrifugation, raising and selection of, and competitive binding assays with, monoclonal antibodies against the receptor. Analysis of ligand binding and molecular mechanisms and models of bioelectrical signal transmission are dealt with in detail as a matter of course.

Some of the other receptors reported on have not as yet been studied farther than at the level of hypotheses. For instance, it is suggested for the psychotropic constituents of *Cannabis sativa* L. that they may act via their own receptors, i.e. specific tetrahydrocannabinol binding macromolecules. This molecule has long been known to be the psychotropic principle of hashish and marihuana, but neither pharmacologists nor neurochemists provided before conclusive evidence concerning its molecular mechanism of action.

Other receptors, such as the beta-adrenergic receptor are visualized on autoradiograms in the book.

The basic questions and techniques for answering them seem to be very similar with all these receptors, so that exchange of ideas and experiences is very useful. Probably this is why strong emphasis is laid in the book on the presentation of a variety of experimental techniques.

The rest of the articles compile different data on individual receptors, reflecting that this area is at a stage of data collection in order to build up a basis on which organization of our knowledge will become possible.

A combined author and subject index at the end of the book helps the reader to find easily what he or she is looking for. This collection of papers appears to be particularly useful to specialists engaged in neurotransmitter, neuroreceptor or membrane receptor research.

P. ARÁNYI

Hormone Antagonists. Edited by M. K. Agarwal. Walter de Gruyter, Berlin, New York 1982, pp. 732.

For the first time, this volume groups under one cover all the hormones for which specific antagonists have been established. Both the conceptional consequences and the clinical use of the various systems are presented. Since the wide spectrum of biological responses, experimental approaches and laboratory technics in one field are often not consulted by workers in another field, one of the greatest advantages of this volume is the fact that it brings together all these diverse ideas and models thus stimulating research in a wider area.

The papers are grouped along three main topics. The first section of the book (24 papers) deals with steroid hormone antagonism. In the second part (10 papers) antagonists against peptide hormones are covered, whereas the last section (5 papers) groups together familiar substances such as antihistamines with those which are just beginning to emerge, e.g. antipheromons, but which are of great importance for both the basic and the industrial research.

One may well expect that the present volume will remain a major reference source to graduate students, research scientists and clinicians for some time to come. This book is value for your money. K. DI GLERIA

Steric Effects in Biomolecules, Proceedings of the International Symposium held in Eger, Hungary, 5–8 October 1981. Edited by G. Nárai-Szabó, Akadémiai Kiadó, Budapest 1982, pp. 417.

This volume contains 27 selected papers presented on the Eger Meeting on Steric Effects in Biomolecules. The aim of the meeting was to provide contacts between scientists working on combined applications of various experimental technics and theoretical calculations. X-ray cristallography, high frequency kinetic nuclear magnetic resonance measurements, circular dichroism studies, QSAR and quantum chemical calculations make up the methodology.

The papers are grouped along three main topics in the book: 14 deal with small molecules, 3 with DNA and RNA, and 10 with various structural and functional aspects of proteins. No doubt, the organisers aim was to cover as much of the field as possible, although for obvious reasons this seems to be a rather difficult task. The editor, on the other hand, decided to give more room for revelant reports in this volume, rather than to include all lectures in a condensed form. This has the advantage of converting this book into a reliable reference book on the topics covered.

J. HAJDU

Electrophoresis 81 by R. C. Allen, P. Arnaud. Walter de Gruyter. Berlin, New York 1981, pp. 1021.

This book presents, on 1021 pages, the proceedings of the Third International Conference on Electrophoresis, held in Charleston, South Carolina, from April 7 to April 10 in 1983. Over 100 presentations, originally given either orally or as posters, are grouped in four sections, entitled, respectively:

I: Theory and Methods
II: High Resolution Two-Dimensional Electrophoresis
III: Biomedical and Biological Applications
IV: Isotachophoresis and Free Flow Electrophoresis

The editors point out that not every presentation fits exactly into one of the above groups, however, the best choice was made in each case.

This volume is invaluable, indeed almost indispensable to researchers in biology and biochemistry, as it forms a handy compendium of the most recent developments and techniques in the separation of biological materials by means of electrophoresis.

The first section covers the wide spectrum of the traditional theory and practice of electrophoresis as well as recent developments in this field. Of particular interest are the techniques of isoelectric focusing on polyacrylamide gels, agarose and cellulose membranes. This section furthermore deals with zone immunoelectrophoretic assay (ZIA), affinity electrophoresis, as well as SDS electrophoresis.

The second section, which concentrates on high resolution two-dimensional electrophoresis, presents a picture of the fields of research where this technique may be applied, for example as a screen for genetic disease markers in humans and plants. Computerized modelling and analysis are also discussed.

Whereas the first section is the most interesting from the point of view of a general overview, the most rewarding section for the researcher is the third, which concentrates on the biomedical and biological applications of electrophoresis. Here one may study the various methods available for the separation of immunoglobulins, lipoproteins, urinary proteins, various isoenzymes, glial tumour cultures for glial markers, hair proteins, nucleic acids, etc.

The final section of the book deals with isotachophoresis of some enzymes, proteins and lipoproteins, as well as free-flow electrophoresis techniques. An electrophoresis program, conducted by NASA, is also reviewed.

Separate author and subject indices are provided and comprehensive bibliography sections are given at the ends of the various articles. This feature should find favour with both researchers and students of the subject. The book is to be recommended also for the numerous monochrome, and some colour illustrations.

E. LINK

Analyse in Mikrobereichen. Neurogene und myogene Erkrankungen der Muskulatur by P. U. Heitz (Acta Histochemica 28) VEB Gustav Fischer Verlag, Jena 1983, 170 figures, 31 tables.

The publication emphasizes the importance of using up-to-date histochemical methods in both research work and clinical diagnostics. The application of histochemical methods extends from routine histopathological diagnostics to molecular biology; thus the volume is necessarily interdisciplinary in character

The chapter dealing with analytical micromethods consists of four lectures: *A. G. Cuello* (Oxford) describes the immunohistochemistry of monoclonal antibodies. The use of antibodies reacting with neuropeptides and neurotransmitters enables us to recognize the anatomical organization and pathological changes of the cells as well as to check their sensitivity to drugs. The radioimmunocytochemistry based on radioactive labelling of antibodies *in vitro* allows quantitative measurements to be made. The author, however, seems to overestimate the importance of the monoclonal immunohistochemical method to that of the traditional method. *G. Pfefferkorn* (Münster) provides a good summary of the histochemical application of wave- and particle-radiation. The highly-sensitive laser method of mass-micromeasurement and the possibility of tissular isotope localization are discussed in detail. Emphasized are the advantages of the laser method over X-ray microanalysis. The method of ionmicroanalysis and that of ultrasound microscopy are also reviewed. The discussion is clear and concise, the figures are vividly descriptive and the tables easy to survey. This is the most conscientious and most detailed part of the volume. By using topochemical and electronwave microprobe methods *Höhling et al.* (Münster) follows the topical and quantitative changes of seven inorganic ions during the process of ossification. *Zierold et al.* (Dortmund) describes how to detect water-soluble substances on ultrathin sections by means of X-ray microanalysis. In the topic of neurogenic and myogenic diseases of the muscles the paper of *K. Kunze* (Hamburg) first recounts the present problems, then *F. Gullotta* (Bonn) discusses the role of enzyme histochemistry. The new methods gave a possibility for the early recognition of certain diseases of the muscles and led to the recognition of numerous new "myopathies", as well. The authors consider these methods as being of importance in encouraging further electronmicroscopic and histochemical investigations. *Goebel et al.* (Göttingen) summarize briefly, but with excellent photo-documentation the importance of electronmicroscopic investigations in recognition of normal and pathologic fibre-structures, with special regard to fibre inclusions. They consider the electronmicroscopic investigations of muscle biopsy samples as unavoidable mainly in the diagnosis of congenital myopathies, myasthenia syndromes, inclusion myositises and mitochondrial myopathies. *U. P. Ketelsen* (Freiburg) discusses the surface scanning-electronmicroscopic investigation of the plasmolemma in myogenic diseases of the striated muscles, a procedure enabling the structural elements of the cell membrane to be analyzed under normal and pathologic conditions. The microparticula observable on the cell membrane show characteristic qualitative and quantitative alterations in certain myogenic syndromes. *Röthig et al.* (Hamburg-Eppendorf) report on the clinico-biochemical analysis of muscle enzymes in diseases accompanied by muscle atrophy. The authors emphasize the importance of muscle loading and repeated investigations. The role of creatinine metabolism and the role of creatininekinase are also analyzed in detail. In the experiments of *Reichmann et al.* (Konstanz) on mice type II fibres were transformed into type I fibres by means of low-frequency stimulation. In the course of this, also the whole enzyme system of the muscle fibres underwent transformation. Since in certain dystrophies type II fibres show increased sensitivity to pathologic processes, the practical value of the question might be a possibility of potential prevention of dystrophic processes.

The chapters of this Supplement of Acta Histochemica dealing with microanalysis and diseases of the muscles consist of papers conscientiously written at high expert level. The articles, inspirative as they are for the beginners, fail, to give them a sufficient basis. For experts they are lacking in most necessary

details; the endeavour to completeness seems to be missing throughout the whole volume. So, the booklet is really helpful only for research workers dealing with special questions: and even this is to be thanked mainly to its abundance in references to the pertaining literature.

The volume contains the material of the 23rd Symposium of the Gesellschaft für Histochemie held in Münster in 1981. The wide spectrum of the application of the histochemical methods is well demonstrated in the volume. The discovery of histochemistry took place at the end of the 19th century, but the peroxidase reaction worked out at that time is still a basic method in haematology. The differentiation of the granulocyte line would have been impossible without this reaction. *Klein* examined the antigens of erythrocytes and glomerular membranes by lectins and monoclonal antibodies. His results help us to interpret the pathogenesis of the most important clinical signs of the haemolytic uraemic syndrome. *Schmalzl* worked out the quantitative determination of some enzymes of the polymorphonuclear neutrophiles by enzyme chemical methods. The publication of *Newman* concerning the leukaemic cell markers and their relationship to the normal cell differentiation is very interesting. The exact characterization of the acute laeukaemias is indispensable to the choice of the treatment. *Budde* analysing the lymphocytes of elderly patients found that the helper T-cells are decreased in elderly women. This could explain the more frequent occurrence of autoimmune diseases in elderly women. *Trost* reviewed the histochemical procedures suitable for the differentiation of lymphocytes in cutaneous lymphoma. *Melzner* examined the binding of low density lipoproteins to the lymphocyte surface. *Bültmann* reviewed very interesting data of membrane defects caused by virus infections. He examined the change of membrane fluidity of human neutrophil granulocytes under the effect of a chemo-attractant and an ECHO-virus. In the volume we can find also a paper of *Makowitzky* on the selective detection of sialic acid by the topooptical method of *Romhányi*. *Schaefer* reported on the congenital and acquired defects of granulocyte peroxidase.

The afore mentioned papers show the widespread application of the histochemical methods in medicine. The publications first seem to be of theoretical-histopathological interest, but on reading them thoroughly one can notice that they give much interesting and important information for the clinical practice, too. We only regret that the figures are not coloured. Beside histopathologists, specialists in haematology, internal medicine or neurology can also get useful information from the book.

J. CZOPF
I. NAGY

Die bakterielle Zellwand (The Bacterial Cell-Wall) by Guntram Seltmann (with the cooperation of Otto Kühnemund and Rolf Reissbrodt). VEB Gustav Fischer Verlag, Jena 1982 pp. 207

In the last decades rapidly growing number of papers have been published on the structure and function of different cell wall components of bacteria, showing significant changes in view regarding the role of bacterial cell wall. In this new aspect the cell wall appears as an active organ, to which special roles in transport functions, virulence and pathogenicity, etc. are attributed.

This comprehensive book is one of the surveys published in the last years. It consists of eight clearly organized chapters. After a short section on morphology, the author describes the structure and biosynthesis of the peptidoglycan rigid layer in Gram positive and Gram negative bacteria. In the third and fourth chapters the components of the outer membrane of Gram negative and Gram positive bacteria were reviewed; among others lipids, lipopolysaccharides, special polysaccharide antigens (e.g. enterobacterial common antigen, teichoic acids, etc.) and different membran proteins (matrix proteins, protein-A etc.). The fifth section deals with the structure and biosynthesis of capsular substances such as the enterobacterial K-antigens, the Vi-antigen, capsular antigens of Staphylococcus, Pneumococcus Haemophylus. Chapter six is a brief description of flagella, fimbriae and pili from morphological and functional points of view. In the seventh chapter some newly proposed models

of Gram negative and Gram positive cell walls were presented. In the eighth section, the author discusses the functions of cell wall, both the static ones i.e. the protective function, the role in bacterial adherence, etc., and the dynamic ones, e.g. transport functions, the role in penetration, etc. This last chapter contains a short treatise on the interactions between cell wall and bacteriophages.

Lists of literature are given separately for each section. Unfortunately, only the main reviews and basic papers are listed which, however, were scarcely referred to in the text. In chapter 3 a very detailed general description of lipids and of protein structure can be found. This style is characteristic for biochemistry textbooks rather than for monographs.

The foreword of this book was dated in October, 1979. Yet, the author brought great care to be up to date: in an appendix, each chapter has a supplement with recent advances in the field covered up to 1981. Despite of these efforts, some deficiencies and mistakes could not be avoided in this book. To mention some examples, a more than 10-year-old table for sugar components of lipopolysaccharides was cited without revision. Therefore, classes of newly detected compounds are missing. Some recent advances in core and K-antigen biosynthesis were not reported, either. In the chapters "Biosynthesis of lipid A" and "Biosynthesis of core" UMP-GlcN and CDP-KDO are incorrectly given for UDP-GlcN and CMP-KDO, respectively. The currence of M-antigen is definitely stated (in chapter 5) for *all* Enterobacteria, albeit, it was found in Escherichia, Klebsiella and Salmonella only. Nevertheless, this book is a very useful summary and source for everybody being active in medicine or biology and showing interest in the biology and biochemistry of the bacterial cell wall.

T. KONTROHR

Abkürzungen in der Medizin und ihren Randgebieten (Abbreviations in medicine and in its adjoining territories) by U. Spranger. VEB Verlag Volk und Gesundheit, Berlin 1980, pp. 240.

Strictly speaking, the index of abbreviations of technical terms arranged in alphabetical order makes 214 pages of the book totalling 240 pages. The rest is constituted, as usual, by the preface, table of contents, directions for use, explanation of different abbreviations and symbols, as well as a list of references. As it is announced by the author, the full forms of 13 500 abbreviations can be found in the book. The abbreviations are collected not only from German medical and biological literature, but also from other languages, mainly from English. The usefulness of the work is greatly increased by the fact that it contains also the new SI units of measure and their conversions. A further merit of the book is, that the abbreviations of the names of several scientific societies can also be found in it.

Collecting and interpreting the abbreviations of technical terms in medicine and in its adjoining territories and putting them into alphabetical order is an undertaking which is worth of respect. It is obvious, however, that the range of this wide variety of entries can be determined only arbitrarily (e.g. is physics or physical chemistry an adjoining territory?). There must be some arbitrariness in deciding whether an abbreviation should be included or not, depending on the currency of its use. This underlines the bravery of the undertaking which is worth of appreciation in any case. In the meantime it must have turned out from the number of copies sold, whether the book was necessary or not. In my opinion it can be useful for everybody who is in connection with medicine or experimental biology. It is a good thing to have this book at hand, but when looking for certain abbreviations one should be prepared for failures in finding some of them, and this can be a source of annoyance. For example, the following abbreviations cannot be found in the book: ACP (acyl carrier protein), ac. (acute), ad vitr. fusc. (ad vitrum fuscum), art. (articulation), bar (10^5 Pa), BLM (bimolecular lipid membrane), c (velocity of propagation of light), c.c. (Concisa, contusa), can (canal), cap. cer. (capsula cerata), caps. operc. (capsula operculata), carb. (carbonic), cer. (cera), cg (centigramm), comm. (communis), corp. (corpus), CoQ (Coenzim Q, ubichinon), desc. (descendent), DT (tolerated dose), E_a (activa-

tion energy), g (gravitation constant), glut. (gluteal), J_r (volume flow), kg (unit of mass), Klysm. (klysma), L (Loschmidt's constant), ln (natural logarithm), L_p (hydraulic conductivity), m (metre), μ (chemical potential), MeV (megaelectronvolt), ml (millilitre), mOsm (milliosmolar), ma (millisecond), nucl. (nucleus), P (probability), Φ (phi, osmotic coefficient), prox. (proximal), ptx (pneumothorax), ρ (ro, density), σ (sigma, reflection coefficient), U (internal energy), ventr. (ventral).

F. VETŐ

Physics of Bioenergetic Processes by L. A. Blumenfeld. Springer-Verlag Berlin, Heidelberg, New York, Tokyo 1983. pp. 119.

This work represents "Volume 16" of the Springer Series in Synergetics. It consists of 119 pages with 30 figures. The book is divided into six chapters among which "Membrane Phosphorylation" and "Proteins as Molecular Machines" are the most conspicuous.

The monograph deals with the principal bioenergetic processes from a physical point of view: muscle contraction, active transport of ions, enzymatic catalysis, oxidative phosphorylation in mitochondria and photophosphorylation in chloroplasts. The theoretical basis upon which this treatment rests is the concept that the physical mechanisms of processes involving highly orded macromolecular structures require, as a rule, not a statistical, but a mechanical approach. The analysis carried out in this book reveals the inadequacy of the classical physicochemical approach to the problems of intracellular energy transduction. This analysis shows that in all bioenergetic processes we are dealing with one and the same phenomenon: energy coupling between two chemical reactions actualized by the help of specific macromolecular constructions, by machines of molecular dimensions. This new approach forms the basis of a physical description of all bioenergetic processes.

This interesting survey can be warmly recommended for biophysicists as well as for biochemists.

I. ALKONYI

Electronic Conduction and Mechanoelectrical Transduction in Biological Materials. Edited by B. Lipinski. Marcel Dekker, Inc., New York and Basel 1982.

This book is a good demonstration of the continual research done in the field of bioelectricity despite the existing skepticism concerning the relevance of the ideas of solid state physics to biology and medicine.

The intention of the editors is that the book should serve not only researchers in the electronic and material sciences but also those in the biological and medical areas. This volume presents an excellent collection of works from the diverse field of bioelectricity thus, it makes accessible to the reader the results of both fundamental and applied research. In addition, a well-balanced description of the theoretical background is given to introduce the basic principles.

The book consists of five chapters written by prominent international contributors. Chapter 1: Electronic Conduction in Biopolymers by R. Pethig; Chapter 2: Biological and Organic Superconduction at Physiological Temperatures by F. W. Cope; Chapter 3: Piezoelectricity of Biological Materials by E. Fukada; Chapter 4: Pyroelectric Effect in Biological Materials by S. B. Lang; Chapter 5: Mechanoelectrical Effects in Biological Systems by N. Guzelsu. All five chapters have similar logic and construction: the theoretical part is followed by critical survey of the existing approaches and results, and finally the conclusion and the perspectives (perhaps the most interesting parts for the readers from biomedical sciences) are given. There is an exhaustive list of references added to each chapter. It is seen throughout the book that the authors tried to arrange the information in a way to facilitate the understanding the particular problems even when they were separated from the whole subject. The topics covered by the book presumably also attract scientists having little practice in advanced mathematics. It is, therefore, desirable to make a good compromise in the presentation of the necessary theoretical basis. This practical point of view seems to be shared by the contributors, though chapter 5 will, probably, discourage some of the readers. For them we suggest to start with the applications of

the methods to biological materials. This part is written in every chapter in a self-consistant and excellent style. The different kinds of biological materials (biomacromolecules, tissues etc.) are discussed separately and in sufficient detail. Unfortunately (perhaps it is inevitable) certain biomaterials receive no attention in the book (e.g. lipids).

This is undoubtedly a revolutionary volume that contains a lot of useful information from a new field of science. It is strongly recommended for all scientists, both optimists and pessimists, who have direct or side interests in electrical phenomena in biology.

I. SZUNDI

Reinigung und Konzentrierung animaler Viren (Purification and Concentration of Animal Viruses) by Herbert Liebermann, VEB Gustav Fischer Verlag, Jena 1982. 141 pages, 45 figures, 16 tables.

In consequence of the rapid development of virology there is an increasing demand for producing large quantities of viruses, purifying and concentrating them and extracting the components in a form appropriate to the aim of utilization. Since until now no monographs have appeared in this topic in German language, this volume remedies a deficiency. The author — who is actually a biophysicist employed in the famous Friedrich Loeffler Institut der Deutschen Akademie der Landwirtschaften, Insel Reims, that deals with animal viruses — designed the monograph for biologist-, veterinary- and medical virologists. Though the subject indicated in the title is the examination of animal viruses, the methods can, naturally, be applied for human-pathogen and plant viruses, too.

Beside the introduction, the monograph has three main chapters. The first chapter, consisting of 10 pages altogether, entitled "Allgemeine Charakteristik der Animaler Viren" (The Characteristics of Animal Viruses) deals with the taxonomy of viruses and briefly reports about the physico-chemical methods used for characterizing them. The treatment of the so called "cryptogram" in this chapter has to be considered antiquated, since, because of its significant deficiencies, ICTV does not recommend it to use in the future. In Table 1 (page 12) the places of Parapoxyvirus and Vaccinia virus have been interchanged. It is also a mistake in taxonomy that Bunya viruses are indicated among DNS viruses in Table 10 (page 70).

The best part of the book is the chapter of 92 pages entitled "Virusreinigung und -konzentrierung" (Purification and Concentration of Viruses), consisting of a general part (80 pages) and a detailed part (12 pages). A detailed survey of simple methods for purification of viruses, the different varieties of column chromatographic methods, the methods for membrane filtering, and a very detailed report of the methods of centrifugation is presented in the general part. Beside this we get appropriate information about other methods of purification too, and also about the methods of concentration. The survey of the methods is so detailed, that even a beginner can acquire expert knowledge by it. When reporting about the operations, the book refers to special literature, indicating the place where these pieces of literature can be found. A particular value of the general part is that, when dealing with the methods of purification and concentration, it touches the possible versions, collates them, offering the research-workers a possibility to chose between alternative ways. It is thought very useful by the reviewer, that — while reporting about the methods — those instruments and chemicals obtainable in socialist countries are taken into account. It was a pleasure to find that e.g. operations realizable with the Hungarian LABOR MIM instruments are also reported about.

In the detailed (special) part some of the well-working methods of purification and concentration of the most important species of the best known families of viruses are presented. They serve practically as models for work with other species of viruses not occurring in this part.

A brief account about obtaining and purifying virus-components (proteins, nucleic acid) is presented in the last chapter consisting of 12 pages. The way of obtaining and purifying proteins and nucleic acids of some viruses (herpes, bovine leucosis virus) are treated in detail. While presenting the methods, the author makes the reader acquainted with his own methods as well as with those obtained in his own institute.

The book is a useful aid for experts working in the field of virology; they are saved a lot of unnecessary quests by having the description of the methods and the possibilities of their application. The clear structure as well as the use of the decimal numbering makes the book easy to survey. The typography is very attractive.

A. BARTHA

Zelluläre Proliferationskinetik by K. J. Halbhuber, J. Kunz and K. J. Stiller (eds), Acta Histochemica, Supplementary volume 27. VEB Gustav Fischer Verlag, Jena 1983

The volume contains the material of the meeting of the Topochemical and Electronmicroscopic Society of GDR held in March, 1981. It consists of 324 pages, 171 figures and 45 tables. Most of the articles are written in German, a smaller part of them in English, with short summaries in English.

The articles, losely connected to each other are thematically divided into 5 chapters. Maure-Schultze describes the basic parameters of cell-kinetics, and the data obtainable with autoradiography, especially those obtainable with double labelling in the chapter entitled *The Bases of Cell-Proliferation*. Beside comparing normal and tumorous cell-proliferation, the connections of the effect of cytostatica to cell-kinetics are analyzed. The determination of the cell-cycle by flow-cytometer and the computer program used in it are described by Krug. From examining the morphological characteristics of cell proliferation David concludes that, within a cell, the proliferative activity and the ability to perform a specific function may be parallel. The factors taking part in regulation were examined in vitro by Sávoly, while Altman ascribes a significance — beside several other events — to the poly-ADP-ribolization of histones in the reconstruction of DNS. In the chapter entitled *Kinetics of Tumour Cells* Matthias comes to the widely accepted conclusion, that there is no close connection between the parameters of cell kinetics and chemotherapic sensitivity. In contrast, several authors try to utilize the in vitro incorporation of precursors (^{3}H-timidine, ^{3}H-uridine) as an indicator to forecast the effectiveness of a chemotherapic treatment. This approximation has been much criticized in the latest years despite that several researchworkers have experienced a connection between the incorporation-inhibition measured in vitro on the one side, and the response reaction of tumours, on the other side. Some papers deal with the cell-proliferation changes caused by carcinogenes (Urban, Danz as well as Amlacher et al.), raising the possibility that the proliferation changes caused by carcinogenes and those ensuing during regeneration are both consequences of the same regulators: the increased activity of endogeneous stimulators of growth. The next chapter deals with the *Cell Kinetics of the Gastro-Intestinal Tract*. Appleton gives a detailed survey of the kinetic characteristics of each section of the digestive canal. Concerning the changes ensuing in pathological states, data can be found for chronic atrophic gastritis as well as for the cell proliferation observable in benign and malignant tumours of the stomach and the large intestine (Steenbeck et al., Theuring et al.). The conclusions of the latter ones, according to which the in vitro autoradiographic examinations can have a role also in the diagnostics of tumours, can be doubted. Among endogeneous regulators the effect of gastrin is examined by Jonas and Barten. The papers collected under the head of *Cell Proliferation of Arterial Wall* are about a factor of possible importance in several pathological events. Thus, in the opinion of Kunz and in accordance with the opinion of others, the artherosclerosis occurring in the wall of arteries can be considered as a special form of wound healing. In experimental hypertension (acute angiotensine hypertension) the proliferation of smooth muscle elements is the leading alteration, in the opinion of Engler et al. Similarly, an increased proliferation activity of smooth muscle elements of the aorta was observed in experimental diabetes (Dénes et al.). In the mechanism of calcification caused by vitamine D_3, Regal ascribes a significance — beside hypercalcaemia — to the increased production of the basic material (proteoglycan) occurring during the action of D_3. Under the title: *Others* examinations concerning the cell proliferation and differentiation of the salivary gland, the cartilaginous tissues and, the myocytes as well as examina-

tions on the role of the cAMP system can be found.

It can be regarded as a merit of the volume, that it presents well-considered works, which give good methodologic descriptions from different areas of research of cell-kinetics. Besides, some short, but good-level works are also included. It is a deficiency, however, especially for those interested in tumours and chemotherapy, that most of the applied methods still determine kinetic parameters of descriptive character, and one finds hardly any examples for a more functional approximation such as the tumour-parent cell problem, the application of clonogen assay, or the heterogenity of tumours. At last, the high-level, careful typographical work, usual in this series, must be underlined.

L. KOPPER

Medizinische und biologische Bedeutung der Thiocyanate (Rhodanine) Edited by W. Weuffen. VEB Verlag Volk und Gesundheit, Berlin 1982. 428 pages, 65 figures, 107 tables, 29 sketches and formulas

In this volume 33 authors, most of them from DDR, but also from Czechoslovakia, Finland, Hungary and USA completed an overview about the biological and medical significance of thiocyanate. According to the introductory chapter the starting point and background of this work was the knowledge accumulated in this field during the last 30 years in the laboratories of the editor and his colleagues. Their main research field and a special scope of this book is the effect of thiocyanate on immune processes and its possible ways of utilization in animal husbandry and medical practice. Furthermore their task was to review all available references which are relevant to the biological actions of this agent.

Six chapters are concerned with the chemical, physiological and pharmacological aspects of thiocyanate. The chemical part (H. Böhland) describes the binding characteristics of NCY groups (Y=O, S, Se, Te) and their determination with modern analytical methods. The pharmacology and toxicology of anorganic thiocyanates form the third chapter which was written by A. Grisk, A. Kramer and W. Weuffen. Three shorter, more specialized chapters cover the following topics: the effect on transmembrane potential of HeLa cells and on mitotic activity (K. Redman), the characteristics of the thiosulphate: cyanid-sulphurtransferase (D. Tanka) and peroxidase-catalysed oxidation of thiocyanate (E. L. Thomas). A brief account of the action of KSCN on the growth of different microorganisms is listed by B. Skokovsky. Three further chapters written by W. Weuffen with coworkers are devoted to describe the scope of analytical methods and to summarize the quantitative data with references about the content of thiocyanate in plants, foods as well as in different parts of experimental animals, domestic animals and men. In chapter 11 the method of thiocyanate determination by means of ion-selective electrodes is described by E. Pungor and Andrea Hrabéczy-Páll. The next chapter is a review on the mechanism of antithyroid effect of thiocyanate (N. Michajlovskij), while the following one written by the coworkers of the Nobel prize laureate A. J. Virtanen (M. Saarivirta, M. Kreula) is concerned with the occurrence of goitre causing substances in food and foodstuff. The original work of W. Weuffen and his associates regarding the action of thiocyanate on immune responses, its tentative utilization in animal husbandry and the blood thiocyanate level in patients having malignant diseases are presented in 5 chapters. Among the complex results presented by the authors, the most remarkable is the enhancement in antibody titer under experimental conditions, which might be responsible for the lower morbity and more economical meat production of calves fed with thiocyanate additive. A table forms the last chapter which contains the chemical structure, name and therapeutic use of about 200 organic and anorganic compounds with $-SCN-$ or $-NCS-$ groups.

The precise documentation of original findings, the thorough collection of about 1000 references and a subject index comprising nearly 2000 items will be very useful information to all scientists who are interested in any biological chemical or medical aspects of thiocyanate. It can be recommended also from a practical point of view to zootechnicians and veterinarians.

J. SZOLCSÁNYI

Hoppe-Seyler's Zeitschrift für Physiologische Chemie

Edited by K. DECKER, W. STOFFEL, G. ZACHAU

Founded in 1877. Continued by A. Kossel, F. Knoop, K. Thomas, F. Lynen, A. Butenandt and G. Weitzel
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