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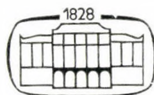

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Rose Bengal as a Tool in Studying the Ligand Binding of Phosphorylase b

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The interaction of rose bengal (RB) with rabbit skeletal muscle phosphorylase b (1,4-α-D glucan: orthophosphate α-glucosyl-transferase, E.C. 2.4.1.1.) was studied by kinetic and absorption photometric methods. RB inhibited the phosphorylase b activity. Inhibition was strictly competitive with respect to substrate G-1-P and activator AMP with inhibition constants 2×10^{-6} M and 2.2×10^{-7} M, respectively. The association of the dye with the enzyme elicited a red shift in the spectrum of RB indicating an apolar binding site. According to difference absorption measurements, the enzyme binds two dye molecules per dimer in the presence and absence of both G-1-P and AMP. Binding constants determined from photometric titrations are consistent with those obtained from kinetic measurements. The present findings allow to carry out detailed kinetic investigations on the activator AMP and substrate G-1-P binding of phosphorylase b.

Introduction

Glycogen phosphorylase is one of the key enzymes in carbohydrate metabolism. Two forms of the enzyme are known, phosphorylase a and phosphorylase b.

It has been well documented that phosphorylase b shows fairly complex nucleotide–protein interactions (Graves, Wang, 1972). The catalytic activity of the enzyme has an absolute requirement for AMP (Green, Cori, 1943). Other nucleotides, such as IMP, CMP, GMP, TMP, UMP, as well as several structural analogues of AMP have proved to activate this enzyme to a lesser or larger extent (Black, Wang, 1970; Mott, Bieber, 1970). ATP is a physiologically important competitive inhibitor of AMP activation (Parmeggiani, Morgan, 1962). These data suggest that the phosphorylase b enzyme and the relevant ligands may serve as a model system for the investigation of nucleotide–protein interactions.

Competition experiments, studies on substrate and inhibitor binding, often provide significant contributions to the understanding of the details and mechanism of enzyme action (Webb, 1963; Yonetani, Theorell, 1964; Yagi, Ozawa, 1960). Kinetic measurements, emission and absorption spectroscopy are also powerful methods for the investigation of interactions between small molecules and enzymes.

Abbreviations: RB, rose bengal.

Unfortunately, in the case of phosphorylase b the lack of suitable probes does not allow to study spectroscopically the ligand binding sites of the enzyme.

RB has previously been applied successfully as a spectroscopic probe for the active site region of horse liver alcoholdehydrogenase (Brand et al., 1967). It has also been shown that RB can bind to other enzymes which also bind nucleotides (Rippa, Picco, 1970; Wu, Wu, 1973). In all these cases the binding of the dye is accompanied by a pronounced difference absorption spectrum. The coincidental binding of RB to sites of nucleotides of other enzymes has directed our attention to this dye as a possible potential spectroscopic probe for the allosteric site of phosphorylase b.

We have investigated the interaction of RB with phosphorylase b to determine whether the dye can act as an inhibitor for the enzyme, and, if so, to determine the equilibrium and kinetic inhibition constants. The experiments presented in this paper demonstrate an interaction between phosphorylase b and RB, that is different from the one leading to photoinactivation (Kamogawa, Fukui, 1975). RB proved to be a competitive inhibitor with respect to AMP and/or G-1-P. Upon binding to enzyme RB shows a large difference spectrum and this spectroscopic change was used to further investigate the action of RB on phosphorylase b.

Materials and methods

Chemicals: Microscopic grade RB was obtained from Fluka and was further purified as described (Brand et al., 1967). After purification it showed only one component by thin-layer chromatography. All other chemicals were of analytical grade and purchased from Reanal Chem. Co. (Budapest, Hungary). Glycogen was prepared from rabbit liver by TCA extraction (Stetten et al., 1956).

Phosphorylase b was isolated from rabbit skeletal muscle according to Fischer and Krebs (1962) with the exception that cysteine was substituted by MEA. The four times recrystallized enzyme was passed through a Sephadex G-25 column immediately before use. The nucleotide content of the eluted enzyme was checked by measuring the A^{260}/A^{280} ratio. This value was below 0.54 in all cases. Using a molecular weight of 185000 (Seery et al., 1967), and $A^{280}_{1\,cm}$ (1%) = 1.32 (Buc, Buc, 1975), the molar concentration of the enzyme calculated from the absorbance at 280 nm was expressed as that of the dimer. Kinetic and photometric experiments as well as gel filtration were performed in 100 mM TRIS-HCl buffer (pH 7.5) containing 33 mM MEA and 1.5 mM EDTA.

Enzyme assay: Assay mixtures contained 16 mM G-1-P, 1 mM AMP, 1% glycogen, and 10^{-7} M enzyme, unless otherwise stated. The incubation was carried out at 30 °C in the dark for 10 minutes. The activity of the enzyme was calculated from the amount of P_i liberated from G-1-P.

Photometric measurements were carried out in a thermostated Beckman Acta CV spectrophotometer using cells of 1 cm light path. All measurements were

performed in thermostated cell compartments at (30 ± 0.2)°C. The slit was adjusted to give 1 nm bandwidth at a wavelength of 545 nm.

The binding data were analyzed according to the equation

$$\frac{r}{c} = Kn - Kr$$

where r is the number of moles of RB bound per mole of enzyme, c is the molar concentration of unbound dye molecules, K is the association constant, and n is the number of binding sites on the enzyme molecule.

Calculations: The kinetic constants for ligands were determined from activity data by non linear regression analysis (Wilkinson, 1961). Regression lines fitting the points of Scatchard graphs as well as the linear replots of inverse horizontal intercepts of Lineweaver – Burk plots were calculated by the linear regression method. Calculations were performed with a Hewlett – Packard 9820/a calculator.

Results

Recently it has been found that the presence of RB resulted in a dye-sensitized photo-oxidation of phosphorylase b upon illumination (Kamogawa, Fukui, 1975). To check whether or not is some interaction between the protein and the dye, resulting in a decrease of the activity of the enzyme, the stability of the enzyme was tested in an environment containing RB. A stock solution was prepared containing 7×10^{-6} M phosphorylase and 4×10^{-5} M RB, and kept at 30 °C in the dark. Aliquots were removed for successive activity determinations at intervals of 10 minutes. During a period of 90 minutes there was no significant decrease of activity. Therefore the activity data used for the determination of kinetic constants will not be biased by inactivation of the enzyme due to the presence of RB.

Results of steady state measurements carried out to determine kinetic parameters are shown in Figs 1 and 2. According to the data in Fig. 1, variation of AMP concentration yielded linear Lineweaver – Burk plots for a set of RB concentrations. Similar results were obtained when G-1-P was the variable ligand (Fig. 2). The points represent experimental data and the lines are calculated from non-linear regression analysis of initial velocity vs ligand concentration data (Wilkinson, 1961) in both Figs 1 and 2.

It has been observed that intermolecular interactions of organic dye molecules in aqueous solutions are often accompanied by changes in their respective absorption spectrum (Owen, Sultana, 1972; Brand et al., 1967; Wu, Wu, 1973). In order to look for direct interactions between RB and the ligands of phosphorylase b we made photometric measurements.

The difference absorption spectra of samples containing RB and a component of the assay mixture were recorded using an RB solution of the same concentration as a reference. There was no spectroscopically observable interaction be-

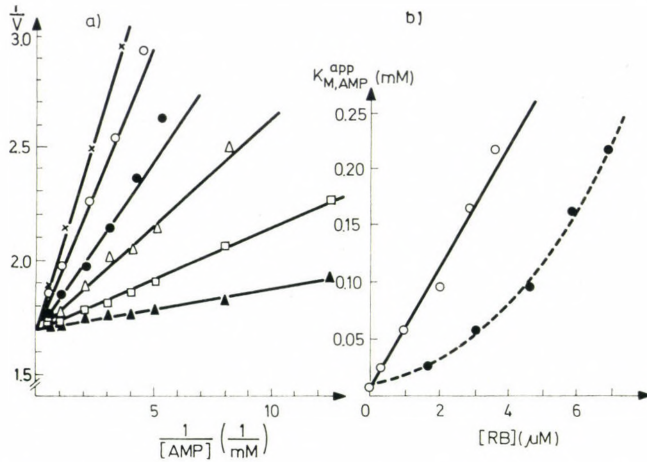

Fig. 1. Plot of the reciprocal of initial reaction velocity (v) vs. the reciprocal of the concentration of AMP in the presence and absence of RB. Concentrations of G-1-P, glycogen and enzyme were held constant at 16 mM, 1 %, and 10^{-7} M, respectively. The concentration of AMP varied from 0.08 mM to 2.0 mM. RB concentrations are none (▲), 1.6 (□), 3.0 (△), 4.7 (●), 6.0 (○) and 7.1 (×) μM. The velocity values are given in arbitrary units. b. Replot of the reciprocal of horizontal intercepts. (●) vs. RB concentration; (○) vs. free RB concentration (for details see the text)

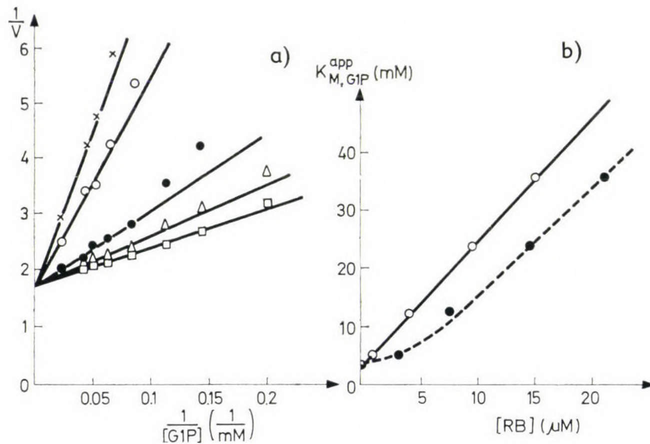

Fig. 2. Inhibition of phosphorylase b activity by RB with respect to varying G-1-P concentrations. The reaction mixtures contained 1 mM AMP, 1 % glycogen, and 10^{-8} M enzyme. The concentration of G-1-P varied in the range 5–48 mM. a RB concentrations are none (□), 3.2 (△), 7.5 (●), 14.8 (○), and 21.8 μM (×). Velocity values are given in arbitrary units. b. Replot of the reciprocal of horizontal intercepts. (●) vs. RB concentration; (○) vs. free RB concentration (for details see the text)

tween AMP and RB and only a slight effect was found between G-1-P and RB. The very small absorption differences measured at three to ten times higher concentrations of G-1-P then those used throughout the present experiments could be increased linearly by increasing the concentration of G-1-P, while keeping the concentration of RB at a constant value of 30 μM (see discussion). Complex formation, however, between glycogen and RB may bring about a substantial decrease of the concentration of the free RB. Scanning the absorbance of a mixed sample of glycogen and RB against the appropriate reference RB solution resulted, after correction for light scattering, in a characteristic difference spectrum with a maximum at 565 nm. The results obtained from photometric titration of a low concentration RB solution with glycogen are presented in Fig. 3 as double reciprocal plots.

The titration was performed at the maximum of the difference spectrum. At high glycogen concentrations, at which the total and free glycogen concen-

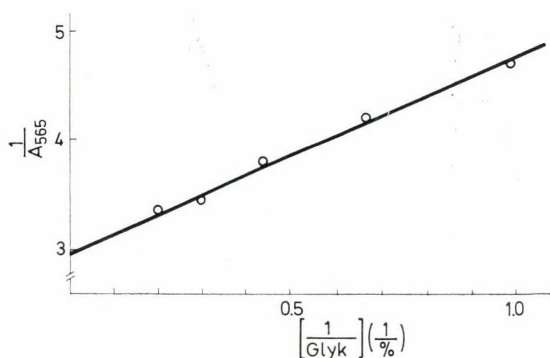

Fig. 3. Titration of an RB solution with glycogen at $\lambda = 565$ nm. The concentration of RB was 5.4 μM. Absorbance values were corrected for the light scattering of glycogen

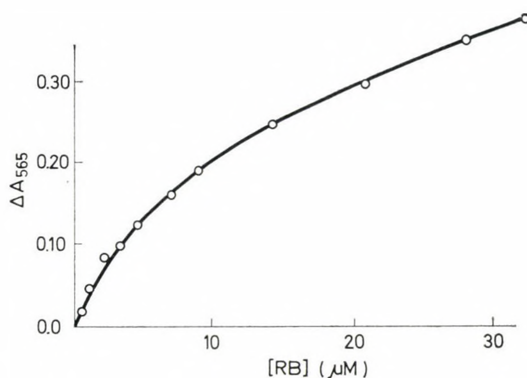

Fig. 4. Titration of a 1% glycogen solution with RB. Difference absorbance values were determined at $\lambda = 565$ nm against an RB solution of the same concentration. Absorbance values were corrected for the light scattering of glycogen

trations were essentially the same, it was possible to fit the experimental points to a straight line. From the ordinate intercept and the applied concentration of the dye a reproducable molar extinction coefficient $\varepsilon^{565} = 8.75 \times 10^4$ M^{-1} cm^{-1} was obtained for the glycogen RB complex. Titration data of a 1% glycogen solution with RB are shown in Fig. 4.

RB has an absorption maximum in an aqueous solution at 545 nm, and in the presence of phosphorylase b the maximum is shifted to a longer wavelength (Fig. 5). The extent of this red shift depends on the relative amount of bound and unbound dye. The difference absorption spectra can be characterized by a maximum unbound dye. The difference absorption spectra can be characterized by a maximum at 563 nm, and a minimum at 541 nm, and by some minor minima at shorter wave lengths (Fig. 6).

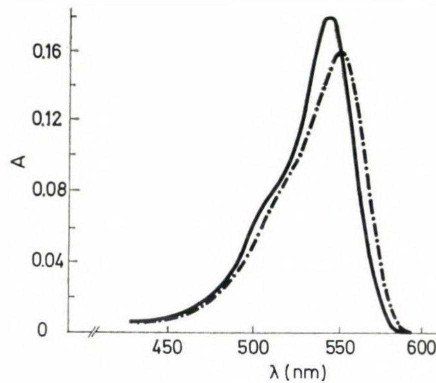

Fig. 5. Interaction of RB with phosphorylase b. Absorption spectra: 2 μM RB (——); 2 μM RB, 0.42 μM enzyme (— · —)

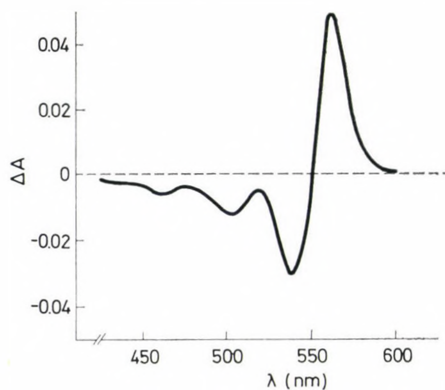

Fig. 6. Difference spectrum of RB bound to phosphorylase be versus free RB. Concentration are the same as in Fig. 5

Titration of enzyme with RB was followed by measuring the amplitude of the difference absorption spectrum at the maximum (Fig. 7). The analysis of the titration data was accomplished by a least squares procedure, using the following equation:

$$K_d = \frac{\left([E]_0 - \frac{\Delta A}{\Delta \varepsilon}\right)\left([RB]_0 - \frac{\Delta B}{\Delta \varepsilon}\right)}{\frac{\Delta A}{\Delta \varepsilon}}$$

where K_d is the dissociation constant, $[E]_0$ and $[RB]_0$ are the total binding site and dye concentration, respectively. ΔA is the measured difference absorbance at 563 nm and $\Delta\varepsilon$ is the molar difference absorption coefficient of the enzyme-RB com-

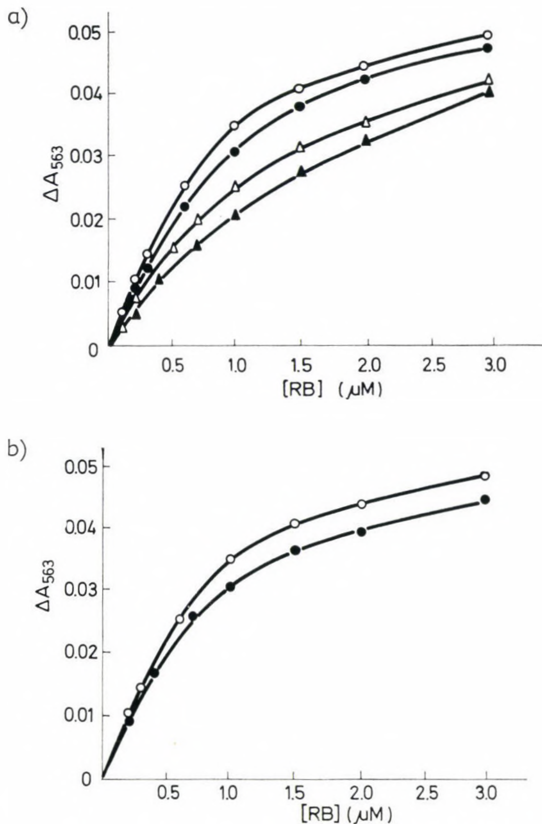

Fig. 7. Saturation of 0.42 μM phosphorylase b with RB, in the presence of AMP and G-1-P. Titration was performed at 563 nm. Difference absorbance values were measured against solutions with the same concentration of RB and ligand but not containing the enzyme. Each titration curve is the mean of five replicates. Additions: a. None (○), 0.1 mM AMP (●), 0.5 mM AMP (△) and 1 mM AMP (▲), b. None (○), and 30 mM G-1-P (●)

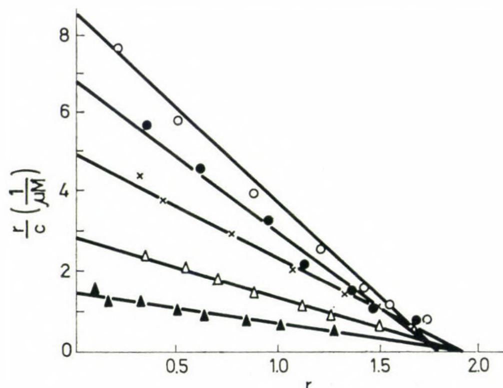

Fig. 8. Effect of AMP and G-1-P on the saturation of 0.42 μM phosphorylase b with RB r and c denote the average number of bound RB molecules per enzyme molecule and the free RB concentration, respectively. The symbols are the same as in Fig. 7

plex at 563 nm. After assuming a value of K_d and $\Delta\varepsilon$, $[RB]_0^{calc}$ values were calculated from the measured ΔA and the $[E]_0$ data. The sum of the squares of the differences between these calculated values of RB concentration and the known values of total RB concentration in solution was then calculated, and this procedure was iterated, varying the K_d and $\Delta\varepsilon$ until the minimum of this sum was found. Analyses were performed for $n = 1$, 2 and 4 binding sites per enzyme molecule (dimer). The best results were obtained at $n = 2$, in all cases. Estimated values of $\Delta\varepsilon$ from the least squares analysis were used for plotting the photometric titration data (Fig. 8) as a Scatchard analysis (Scatchard, 1949). The number of RB binding sites per enzyme dimer was very close to 2 in all the cases investigated.

Discussion

According to steady state kinetic measurements (Figs 1a and 2a), RB efficiently inhibited the activity of phosphorylase b. The inhibitor did not alter the V_{max} of the enzyme reaction, however, it decreased the binding of activator AMP as well as that of substrate G-1-P. Such findings are characteristic of competitive inhibition. Replots of the inverse horizontal intercepts obtained from the Lineweaver – Burk plots versus inhibitor concentrations, however, did not yield straight lines (dotted lines in Figs 1b and 2b) and the K_i inhibition constants evaluated from experiments similar to those shown in Figs 1a and 2a depended on the concentration of RB. Higher RB concentrations resulted in smaller K_i-s.

The curvature of the replots could be interpreted by assuming that the inhibition was due to an associated form of the dye. The concentration of such a polymeric form would show a faster than linear rise with increasing total RB concentration. To test the validity of such an explanation, RB absorption spectra were

measured in the concentration range applied in the above experiments. As no spectral change was observed, and, moreover, since the concentration and absorbance data obeyed the Lambert – Beer Law, this explanation could not be accepted.

If some components (besides the enzyme) of the assay mixture could form a complex with RB, it would yield "anomal replots". Glycogen proved to be the only ligand which was able to decrease the amount of the unbound inhibitor to a measurable extent. The data in Fig. 4 together with the estimated molar absorption coefficient of the RB–glycogen complex, allowed us to make corrections for the amount of this complex in the reaction mixtures. After such correction the replots became linear (solid lines in Figs 1b and 2b). The average K_i value from 12 independent sets of activity assays were 2.2×10^{-7} M and 2×10^{-6} M with AMP and G-1-P as variable ligands, respectively.

Cooperativity in the binding of the dye could also account for curved replots. As the experimental data may be interpreted without taking such phenomena into consideration, we think that cooperativity, if any, cannot play an important role in the concentration range tested.

According to the least squares analysis applied for the determination of molar difference extinction coefficients and the Scatchard plots of the photometric titration experiments (Fig. 8), there are two binding sites per dimer enzyme molecule, either in the presence or absence of AMP or G-1-P. This finding is in accordance with a competition of RB and the two above mentioned ligands on each protomer.

We found that AMP and G-1-P delay the saturation of the enzyme with RB. The values of $K_{d, AMP}$ and $K_{d, G\text{-}1\text{-}P}$ estimated from the increase of the apparent dissociation constant of the RB–enzyme complex are 0.3 mM and 90 mM, respectively.

These data seem rather high compared to the appropriate K_M values of kinetic measurements (10^{-5} M and 3.4×10^{-3} M for AMP and G-1-P, respectively). However, it is known that there exists a pronounced positive heterotrop allosteric interaction between the AMP and G-1-P sites (Graves, Wang, 1972). This would mean that the dissociation constant of either AMP or G-1-P is higher in the absence of the other ligand than in its presence. Our values are about three times higher than those of Oikonomakos et al., (1979) determined in somewhat different buffer at pH 7.0, i.e., closer to the pH optimum of the enzyme.

Photometric and activity measurements showed that RB and phosphorylase b ligands (AMP or G-1-P) mutually hindered each other's binding. This fact may be a consequence of a true competition or of induced conformational changes. In our case a conformational change of phosphorylase b induced by RB could result in a lower affinity of the enzyme to the ligands. Similarly, the binding of ligands might induce a conformational change of the enzyme, decreasing its ability to bind RB.

The values of the dissociation constants of RB–phosphorylase b complex, determined by photometric titration (2.1×10^{-7} M), correlate well with those calculated from the increase of the $K^{app}_{M, AMP}$ in the presence of RB (2.2×10^{-7} M)

The dissociation constant of RB determined from its ability to hinder the formation of the enzyme–AMP complex was an order of magnitude smaller than that determined from its effect on the enzyme–G-1-P binding.

If one accepts that the inhibition phenomena in RB and ligand binding are due to true competition the above findings can be explained by supposing a more complete coincidence of the RB binding site with that of AMP. In the case of the alternative interpretation, although the conformational change induced by RB or AMP totally prevents the binding of the other, the dye's binding would not cause a conformational change completely inhibiting G-1-P binding.

The inhibition of RB binding by both AMP and G-1-P makes possible to carry out detailed kinetic investigations on the activator AMP and substrate G-1-P binding of phosphorylase b no matter whether the inhibition is due to true competition or induced conformational changes. As the complex formation between RB and enzyme is accompained by a change in the fluorescence parameters of the dye (Trón et al., unpublished results) these investigations may apply to both photometric and fluorescence techniques.

We are indebted to Professor S. Damjanovich for valuable discussions. The skillful technical assistance of Miss K. Szováti is gratefully acknowledged.

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Studies on the Structure of Rat Liver Messenger Ribonucleoprotein

I. Isolation and Characterization

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Messenger ribonucleoprotein (mRNP) was released from 0.5 M KCl washed rat liver polyribosomes after mild pancreatic ribonuclease (EC 3.1.27.5) and EDTA treatment and separated by sucrose gradient centrifugation from ribosomal subunits. The method yielded partially fragmented mRNP, which, however, was free from ribosomal contaminants. In CsCl gradient the mRNP banded at 1.46 g/cm^3, indicating a protein content of about 65%. Treatment of mRNP with 0.25 M or 0.5 M KCl resulted in loss of the proteins. Urea/sodium dodecyl sulfate polyacrylamide gel electrophoresis of mRNA bound proteins showed that the most prominent polypeptides found in the mRNP fractions exhibited molecular weights of 29 000 (P29), 31 000 (P31), 38 000 (P38), 44 000 (P44), 50 000 (P50), 54 000 (P54), 63 000 (P63), 76 000 (P76) and 105 000 (P105). Three polypeptides, P38, P44 and P63 were most sensitive to high salt treatment.

Introduction

In eukaryotes mRNA was found to be associated with proteins as messenger ribonucleoprotein (for review see Williamson, 1973; Preobrazhensky, Spirin, 1978). The mRNP could be liberated from the polysomes by EDTA (Perry, Kelley, 1968; Henshaw, 1968) or by puromycin-high salt treatment (Blobel, 1972) and recently also by Cs_2SO_4 gradient centrifugation (Greenberg, 1977; Liautard, Liautard, 1977). The protein composition of mRNP isolated from a variety of cell types has been analyzed and several polypeptides ranging in molecular weight from about 30 000 to 130 000 have usually been detected. Two of them with molecular weights of approximately 52 000 and 78 000 appeared to be universal mRNP components (Preobrazhensky, Spirin, 1978).

In this and our next paper we give a description of the isolation and a detailed characterization of rat liver mRNP. The method used yielded partially fragmented mRNP, which, however, was free from ribosomal contaminants. In the present study we report the protein content and composition of mRNP. It will be shown, that in rat liver mRNP there are nine major and five minor structural polypeptides and that a large proportion of three of them can be removed by 0.5 M KCl treatment of the isolated mRNP.

Abbrevations: mRNP, messenger ribonucleoprotein.

Materials and methods

Materials

[2,8-^{3}H]-adenine (spec. act. 946 mCi/mmol) was obtained from the Isotope Institute of the Hungarian Academy of Sciences; [5-^{3}H]-orotic acid (spec. act. 11 Ci/mmol) from UVVVR (Prague, Czechoslovakia); dithiothreitol from BDH (Poole, England) and all other chemicals from REANAL (Budapest, Hungary).

Buffers

(A) 50 mM Tris-HCl, 25 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.5; (B) 20 mM triethanolamine, 25 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.5; (C) 0.15 M KCl, 20 mM EDTA, 10 mM triethanolamine, 1 mM dithiothreitol, pH 7.5; (D) 10 mM Tris-HCl, 1 mM EDTA, 0.1 M NaCl, 0.5% sodium dodecyl sulfate, pH 7.4.

Preparation of polyribosomes

The labelling and preparation of total liver polyribosomes by a modified method of Blobel and Potter (1967) has been described (Tomcsányi et al., 1973, 1976). In the present version the animals received 100 μCi [5-^{3}H]-orotic acid/100 g body weight instead of [^{14}C]-orotic acid. The polysome pellet was resuspended in buffer A, adjusted to about 100 A_{260} units/ml, then 2.5 M KCl was added to the suspension to a final concentration of 0.5 M, layered onto 3 ml of 1 M sucrose containing 0.5 M KCl, 20 mM triethanolamine (pH 7.5), 1 mM dithiothreitol, 5 mM $MgCl_2$ and centrifuged at 50 000 rpm ($145\,000 \times g_{av}$) for 1.5 hours in a 8×11 ml fixed angle rotor of a Janetzki VAC 601 ultracentrifuge. The pellet was used immediately or stored at -20 °C.

Preparation of unfragmented and partially fragmented mRNP

KCl washed polysomes were suspended in buffer B adjusted to about 100 A_{260} units/ml, 0.25 M EDTA was added to 20 mM/ml and about 50 A_{260} units of EDTA treated polysomes were layered onto 32 ml of 5–45% sucrose gradients in buffer C and centrifuged at 26 000 rpm for 3 hours in the SW 27 rotor of a Beckman L2–65B ultracentrifuge. When fragmented mRNP was prepared, pancreatic RNase was added to a final concentration of 2 ng/ml and incubated at 37 °C for 15 minutes. After cooling and adding EDTA, about 300 A_{260} units of EDTA treated polysomes were layered onto 32 ml of 15–30% sucrose gradients in buffer C and centrifuged at 26 000 rpm for 24 hours in the SW 27 rotor. The 90–140*S* (Fig. 1a) or 12–21*S* (Fig. 1b) regions of the gradients were pooled, precipitated with two volumes of ethanol (24 hours, -20 °C) or, after dilution with buffer C, the mRNP was sedimented at 50 000 rpm ($145\,000 \times g_{av}$) in a 8×11 ml fixed angle

rotor for 5 hours in the presence or absence of 0.25 M or 0.5 M KCl and used for further analysis.

CsCl gradient centrifugation of mRNP

Sedimented or alcohol precipitated mRNP samples were resuspended in buffer B in the presence of 0.01% Triton X-100 and fixed with 4% formaldehyde for 4–16 hours. Of the fixed mRNP samples, 0.2–0.3 ml was layered onto CsCl gradients composed of 2.3 ml of 1.66 g/cm^3 and 2.3 ml of 1.33 g/cm^3 CsCl solutions and centrifuged at 40 000 rpm at 10 °C for 20 hours in the SW 65L rotor. Density was calculated from refractive index measurements.

Determination of acid insoluble radioactivity

Aliquots of 50 to 100 μl were put onto 3 × 3 cm squares of Whatman 3 MM filter paper and dried. The squares were washed with 10 ml of 5% trichloroacetic acid and 10 ml of 95% ethanol and again dried and counted in 10 ml of a toluene based scintillation cocktail in a Beckman LS 230 liquid scintillation spectrometer.

RNA extraction

Alcohol precipitated samples from the 12–21*S* zone of the sucrose gradient (Fig. 1b) were dissolved in buffer D, and RNA was prepared according to Perry et al. (1972).

Polyacrylamide gel electrophoresis of RNA derived from 12–21S mRNP

RNA derived from 12–21*S* mRNP was electrophoresed in 3% polyacrylamide gels (6 cm long) according to Hirsch and Penman (1974). The gels were frozen, then cut into 2 mm slices, each of which was put in a scintillation vial. To every slice, 0.5 ml Tissue Solubilizer (Eastman) was added and the vial kept at 37 °C for 18 hours. Then 10 ml of a toluene based scintillation cocktail was poured into each vial, and radioactivity was measured.

Polyacrylamide gel electrophoresis of mRNP proteins

The polypeptides of mRNP were separated by urea/sodium dodecyl sulfate polyacrylamide gel electrophoresis. The alcohol precipitated or sedimented mRNP samples were washed with 5% trichloroacetic acid, 70% ethanol containing 0.1 M sodium acetate, 70% and 96% ethanol, then dried and dissolved in 1% SDS/1% 2-mercaptoethanol/10 mM sodium phosphate/8 M urea, pH 7.1. Urea/SDS polyacrylamide gel electrophoresis was performed as described by Molnár and Samarina (1975). The gels were stained with Coomassie brilliant blue.

The absorbance profiles of the stained gels were recorded at 620 nm using an automatic Joyce – Loebl Chromoscan. The molecular weights of the polypeptides were calculated accroding to Weber and Osborn (1969) using chimotrypsinogen (M_r 25 000), ovalbumin (M_r 45 000) and bovine serum albumin (M_r 68 000) as molecular weight standards.

Results

Figure 1a shows the sedimentation profile of the EDTA treated polysomes. The radioactivity was distributed in the 10 – 140*S* region of the gradient, which is characteristic of rat liver mRNP (Henshaw, 1968). Treatment of polysomes with a low concentration of pancreatic RNase before EDTA dissociation produced

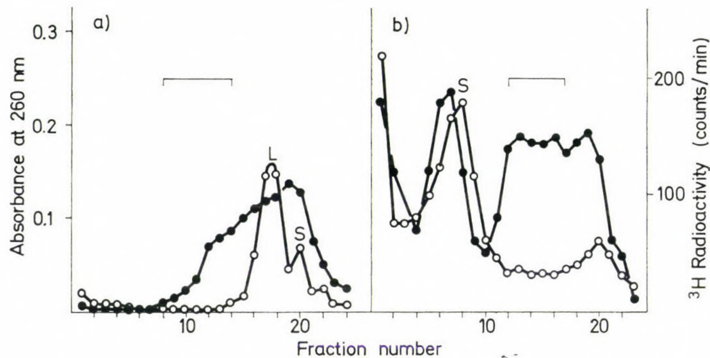

Fig. 1. Sucrose gradient centrifugation of unfragmented and partially fragmented mRNP. [^{3}H]-orotic acid labelled, KCl washed rat liver polysomes were treated with EDTA or with pancreatic RNase and EDTA and centrifuged as described in "Methods". (a) Polysomes treated with EDTA (5–45% sucrose gradient, SW 27 rotor, 26 000 rpm for 3 hours). (b) Polysomes incubated in the presence of 2 ng/ml of pancreatic RNase before EDTA treatment (15 – 30% sucrose gradient, SW 27 rotor, 26 000 rpm for 24 hours). Aliquots of 50 μl were used for the determination of absorbance and radioactivity. The fractions, indicated were pooled and used for further analysis. ○——○, absorbance; ●——●, [^{3}H]-acid insoluble a radioactivity; L, large ribosomal subunit; S, small ribosomal subunit

fragments of rat liver mRNP smaller than 30*S*, which could be separated from the small ribosomal subunits (Fig. 1b).

When the 90 – 140*S* material from EDTA dissociated polysomes was fixed and subjected to CsCl gradient centrifugation most of the radioactive material had a buoyant density of 1.46 g/cm^3 (Fig. 2a). Some material that banded at 1.59 g/cm^3 was presumed to be contamination caused by large ribosomal subunits. The material derived from the 12 – 21*S* region of sucrose gradient centrifugation of RNase and EDTA treated polysomes banded in CsCl gradients as a discrete

peak with a bouyant density of 1.46 g/cm^3 (Fig. 2b). This density is characteristic of mRNP (Preobrazhensky, Spirin, 1978) and corresponds to a protein content of about 65% (Spirin, 1969). Treatment of 12–21*S* mRNP with 0.25 M KCl resulted in the appearance of another radioactive peak at 1.49 g/cm^3 (Fig. 2c). This means that about 25% of the protein content of some of the mRNP was lost. When 12–21*S* mRNP was sedimented in the presence of 0.5 M KCl and analyzed in

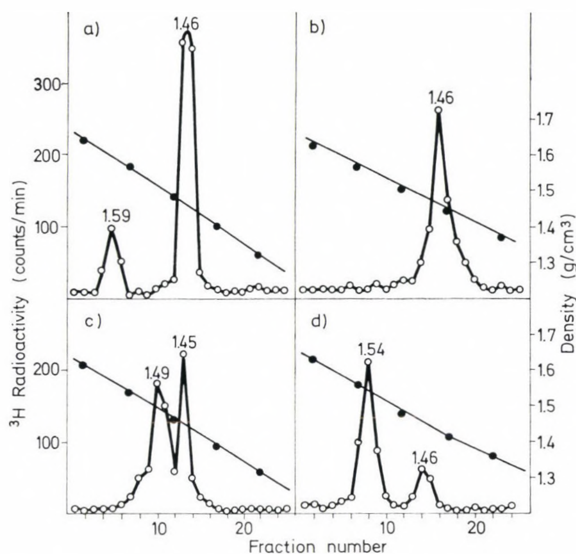

Fig. 2. CsCl gradient centrifugation of mRNP. Alcohol precipitated or sedimented mRNP samples were suspended in buffer B in the presence of 0.01% Triton X-100, fixed with formaldehyde and centrifuged in CsCl gradients at 10 °C at 40 000 rpm for 20 hours in the Spinco SW 65 L rotor. (A) 90–140*S* mRNP from Fig. 1a. (b) 12–21*S* mRNP from Fig. 1b. (c) 12–21*S* mRNP treated with 0.25 M KCl. (d) 12–21*S* mRNP treated with 0.5 M KCl. ○—○, [^{3}H]-acid insoluble radioactivity; ●—●, density of CsCl

CsCl gradients it banded at 1.54 g/cm^3 but a small proportion of mRNP was unaffected (Fig. 2d). Comparison of the density of 0.5 M KCl treated mRNP with that of the untreated mRNP showed that about 50% of the proteins was released as the result of 0.5 M KCl treatment. If the material, derived from the 7–11 zone of the sucrose gradient, was centrifuged in CsCl, two other peaks at 1.53 g/cm^3 and 1.41 g/cm^3 appeared (data not shown).

The polypeptides associated with mRNA were analyzed by urea/SDS polyacrylamide gel electrophoresis. Nine major and five minor polypeptide bands were observed in the 29 000–130 000 molecular weight region with the dominance of a M_r 38 000 polypeptide (Fig. 3a). Polypeptides P29 (29 000), P31 (31 000), P38 (38 000), P44 (44 000), P50 (50 000), P54 (54 000), P63 (63 000), P76 (76 000)

and P105 (105 000) stained intensively in all mRNP preparations. Other bands, notably P58 (58 000), P71 (71 000), P80 (80 000), P90 (90 000) and P130 (130 000) stained with varying intensity but were present as detectable components. The effect of KCl on the protein composition of mRNP is shown in Figs 3b, c and d. While 0.25 M KCl caused a loss of P38 (Fig. 3b), 0.5 M KCl markedly decreased the amounts of P38, P44 and P63 (Fig. 3c) and these removed polypeptides were present in the supernatant after sedimenting the 0.5 M KCl treated mRNP particles (Fig. 3d).

The size distribution of RNA derived from the 12–21*S* region of a sucrose gradient was examined by polyacrylamide gel electrophoresis. As seen in Fig. 4, RNA from the 12–21*S* mRNP contained fragments ranging from about 90 to 1200 nucleotides in length.

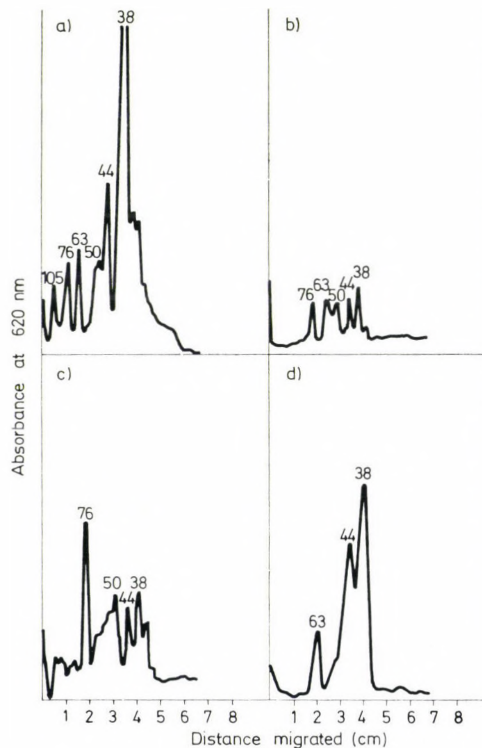

Fig. 3. Urea/SDS polyacrylamide gel electrophoresis of 12–21*S* mRNP. 12–21*S* mRNP samples were obtained from sucrose gradients and treated with different concentrations of KCl. Proteins as described in the "Methods" were prepared for electrophoresis and electrophoresed in 8% polyacrylamide gels containing 0.1% SDS and 6 M urea. (a) Proteins from 12–21*S* mRNP. Untreated, 6 mA/tube, 5 hours. (b) 12–21*S* mRNP treated with 0.25 KCl, 7.5 mA/tube, 8 hours. (c) 12–21*S* mRNP treated with 0.5 M KCl, 8 mA/tube, 8 hours. (d) Proteins from the supernatant of 0.5 M KCl treated 12–21*S* mRNP, 6 mA/tube, 5 hours

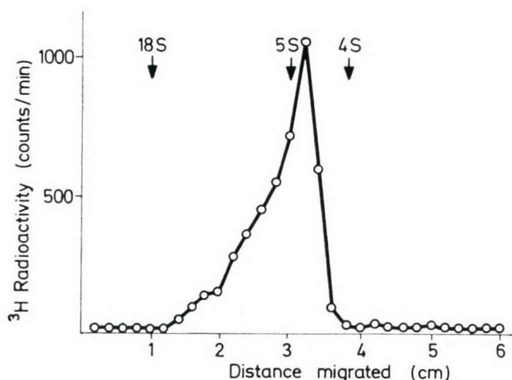

Fig. 4. Polyacrylamide gel electrophoresis of RNA derived from 12–21*S* mRNP. RNA was prepared from 12–21*S* mRNP by phenol-chloroform extraction (Perry et al., 1972), dissolved in 0.2% SDS, 2 mM EDTA 20 mM NaH_2PO_4, 40 mM Tris-HCl (pH 7.4), 10% glycerol and heated at 75 °C for 5 minutes before application onto the surface of 3% polyacrylamide gels prepared according to Hirsch and Penman (1974). The gels were run at 5 mA/tube for 80 minutes, then frozen, sliced and counted as described in the "Methods". Heat treated [^{14}C] labelled cytoplasmic RNA was run in separate gels

Discussion

The methods usually applied for the isolation of mRNP, such as oligo (dT) cellulose chromatography (Irwin et al., 1975; Jeffery, 1977), Cs_2SO_4 density gradient centrifugation (Greenberg, 1977; Liautard, Liautard, 1977) use salt concentrations much higher than physiological. As shown in this paper, exposure of mRNP to high salt removes proteins bound to mRNA (Figs. 2d and 3c). Therefore, we isolated the mRNP at a physiological salt concentration. Although this method yielded partially fragmented mRNP, its properties did not differ from those of undegraded mRNP (Fig. 2a and b). As judged from isopycnic banding, 12–21*S* mRNP is free from ribosomal contaminants and the method allows its preparation on a relatively large scale.

The findings according to which 90–140*S* and 12–21*S* mRNP banded in a narrow density range indicate that there is no large variation in the RNA: protein ratio between mRNPs of different sizes.

Electrophoresis of 12–21*S* mRNP in urea/SDS polyacrylamide gels revealed nine major and five minor polypeptides. Six of them with molecular weights above M_r 50 000, namely, P50, P54, P58, P63, P76 and P90, were found to bind to the poly(A) segment of rat liver mRNA (Tomcsányi et al., 1981a). This suggests that other sequences may also serve as protein binding sites. This will be described and discussed in the following paper (Tomcsányi et al., 1981b).

The predominant polypeptides present in rat liver mRNP appeared to be P38 and P44. These proteins as well as P29 and P31 are in the same range as

ribosomal proteins, but their ribosomal origin seems to be unlikely for two reasons: i) The mRNPs isolated by means of Cs_2SO_4 gradient centrifugation from mouse L cells (Greenberg, 1977) or HeLa cells (Liautard, Liautard, 1977) also contain two polypeptides with molecular weights of 30 000 and 36 000. Under these conditions nearly all of the proteins from the rRNA are removed. ii) EDTA treatment of active rat liver ribosomal subunits partially releases 13 proteins from the 40*S* subunit (Terao et al., 1975) but the molecular weights of none of these agree with those of mRNP proteins. From the 60*S* subunit EDTA removes only one protein (L3) with a molecular weight of 38 000 but this protein is bound to 5*S* RNA, sediments at 7*S* and exhibits an RNA: protein ratio of 1 : 1 (Terao et al., 1975). Indeed, this RNP (1.53 g/cm^3) is present in the 7*S* region, but never in the 12–21*S* region of the sucrose gradient. More recently Orbig et al., (1979) have reported the protein composition of the mRNP of L-cells isolated by using three different methods. They consistently found polypeptides with molecular weights of 32 000, 34 000, 39 000 and 42 000 in addition to the poly(A) bound polypeptides. Our results are in agreement with theirs.

Treatment of mRNP with 0.25 M and 0.5 M KCl showed that most but not all of the mRNP particles had lost a portion of their proteins. These results are in accordance with those obtained by CsCl isopycnic banding of mRNA labelled, high salt and EDTA treated KB cell polysomes (Kumar, Lindberg, 1972) or rat liver polysomes (Cardelli, Pitot, 1977).

The different salt sensitivity of the proteins bound to mRNA was demonstrated by polyacrylamide gel electrophoresis, too. This finding was not totally unexpected since our mRNP preparation derived from total polysomes. Rat liver cells contain about 24 000 different $poly(A)^+$ mRNA sequences in three abundance classes (Savage et al., 1978). Comparison the high number of mRNA species with the low number of proteins found in mRNP suggests that similar group of proteins is associated with different mRNA molecules. However, as shown by the KCl treatment ,the strength of the binding of the same proteins to different mRNA species is not identical because none of them was completely removed from the mRNP.

The protein composition of rat liver mRNP reported here is somewhat different from that obtained by Cardelli and Pitot (1977). They found polypeptides with molecular weights above 50 000, which is characteristic of a poly(A)-protein complex. One of the reasons of this discrepancy may be that the methods used by them were different from ours. These authors isolated them RNP by oligo(dT) cellulose chromatography and elution was done with formamide. Formamide is known to be a dissociating agent, which partially (Lindberg, Sundquist, 1974) or totally (Cardelli, Pitot, 1977) separates proteins from mRNA. Precipitation of mRNP in the presence of a dissociating agent may cause loss of proteins. When 12–21*S* mRNP was precipitated with alcohol in the presence of formamide, P38 and P44 almost completely disappeared (unpublished observation).

The function of mRNP proteins being unknown as yet, their designation as genuine mRNA associated factors must remain tentative.

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Studies on the Structure of Rat Liver Messenger Ribonucleoprotein

II. Non-poly(A) RNase Resistant Fragments and Electron Microscopic Appearance of mRNP

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Partially fragmented 12—21*S* rat liver messenger ribonucleoprotein (mRNP), labelled either with [^{3}H]-orotic acid or [^{3}H]-adenine was treated with 5 μg/ml or 0.1 μg/ml pancreatic ribonuclease (EC 3.1.27.5.) and the resistant fragments were separated by sucrose gradient centrifugation. Two types of fragments were obtained. Digestion of mRNP with ribonuclease at a concentration of 5 μg/ml resulted in 9*S* poly(A)-protein particles of mRNP as evidenced by their characteristic sedimentation, electrophoretic mobility of the RNA moiety and protein composition. In contrast, ribonuclease at a concentration of 0.1 μg/ml produced 2—5*S* pyrimidine labelled fragments carrying polynucleotide sequences consisting of approximately 16—50 residues. Two polypeptides of rat liver mRNP with molecular weights of 38 000 (P38) and 44 000 (P44) were found to be attached to these sequences. The data demonstrate that RNA sequences other than poly(A) interact with protein in mRNP.

Electron microscopic studies revealed that the liberation of mRNP from the polysomes by EDTA changes its surface properties since EDTA releases the mRNP mainly in the form of globular particles. However, a small proportion of the mRNP remains in fibril-like form. Along the fibrils several blobs could be seen which were probably the proteins attached to the mRNA. From the available data a model for the structure of an "average" mRNP molecule is proposed.

Introduction

Eukaryotic polysomal mRNA appears to be associated with a specific set of polypeptides and it is generally accepted that its 3' poly(A) segment serves as one of the protein binding sites (for review see Preobrazhensky, Spirin, 1978). The existence of other sequences involved in protein interaction has however, indirectly been inferred from the occurrence of poly(A) lacking mRNA species in mRNP (Sonensheim et al., 1976; Greenberg, 1977). In the preceding paper (Tomcsányi et al., 1981b) the isolation and characterization of rat liver mRNP were described. The present study reports the separation and partial characterization of non-poly(A) RNase resistant RNP fragments obtained by mild pancreatic RNase treatment of rat liver mRNP. It is demonstrated that two polypeptides of

Abbreviations: mRNP, messenger ribonucleoprotein.

rat liver mRNP with molecular weights of 38 000 (P38) and 44 000 (P44) are found in these fragments.

Electron microscopic studies revealed that in rat liver mRNP the mRNA is not evenly coated with proteins but rather that the proteins, as packages are attached to mRNA, at specific points. On the basis of the available data a model is proposed for the "average" rat liver mRNP molecule.

Materials and methods

[2,8-^{3}H]-adenine (spec. act. 946 mCi/mmol) was purchased from the Isotope Institute of the Hungarian Academy of Sciences, [5-^{3}H]-orotic acid (spec. act. 11 Ci/mmol) from UVVVR (Prague), dithiothreitol from BDH (Poole, England) and all other chemicals from Reanal (Budapest).

Buffers

(A) 0.1 M NaCl, 20 mM triethanolamine, 2 mM $MgCl_2$, 2 mM dithiothreitol, pH 7.5; (B) 10 mM Tris-HCl, 1 mM EDTA, 0.1 M NaCl, 0.5% sodium dodecyl sulfate, pH 7.4.

Preparation of polyribosomes and 12–21S mRNP

Labelling and preparation of polyribosomes and of 12–21*S* mRNP have been previously described (Tomcsányi et al., 1976; Tomcsányi et al., 1981b).

RNase treatment of 12–21S mRNP

Alcohol precipitated or sedimented [^{3}H]-orotic acid or [^{3}H]-adenine labelled 12–21*S* mRNP samples were resuspended in buffer A, adjusted to a concentration of about 10 A_{260} units/ml. Pancreatic RNase was added to the mRNP to a final concentration of 5, 1 or 0.1 μg/ml and incubated at 37 °C for 30 minutes. The RNase treated samples were layered on 32 ml of 5–20% sucrose gradients in buffer A and centrifuged at 26 000 r.p.m. for 24 hours in the SW 27 rotor. The 7–12*S* and 2–5*S* regions of the gradients were pooled, precipitated with two volumes of ethanol and used for RNA extraction or for the preparation of protein for gel electrophoresis.

RNA extraction

Alcohol precipitated samples from the 7–12*S* and 2–5*S* zones of the sucrose gradients were dissolved in buffer B. RNA was prepared according to Perry et al. (1972) and precipitated in the presence of carrier rat liver tRNA with two volumes of ethanol.

Polyacrylamide gel electrophoresis of RNA fragments

Electrophoresis of RNA fragments in 3% and 10% polyacrylamide gels (6 cm long) was carried out according to Hirsch and Penman (1974) as previously described (Tomcsányi et al., 1981b).

Polyacrylamide gel electrophoresis of polypeptides bound to mRNA fragments

Alcohol precipitated samples from different regions of sucrose gradients were prepared for electrophoresis and electrophoresed in urea/SDS gels as described (Tomcsányi et al., 1981b).

Electron microscopy of mRNP

Fractions of the 12–21*S* region of the sucrose gradient (Tomcsányi et al., 1981b) were pooled and prepared for electron microscopy as described earlier (Komáromy et al., 1975). In some experiments sedimented mRNP was fixed with 2.5% glutaraldehyde for 2 hours and then with 2% buffered osmium tetroxide. The dehydrated material was embedded in Durcupan ACM (Fluka, Switzerland). Ultrathin sections were prepared with an LKB ultramicrotome and stained with uranyl acetate and lead citrate. The electron microscopic studies were carried out with a TESLA BS 613 electron microscope (accelerating voltage, 80 kV).

Results

In order to decide which polypeptides are bound to the different regions of mRNA, labelled mRNP samples were subjected to low and high pancreatic RNase digestion. The RNase resistant fragments were separated by sucrose gradient centrifugation and their polypeptide and RNA moieties were analyzed by polyacrylamide gel electrophoresis. In the case of [^{3}H]-orotic acid labelled mRNP 5 μg/ml of RNase resulted in very low level of acid insoluble radioactivity in the 2–5*S* zone of the gradient, while 0.1 μg/ml of RNase produced 2–5*S* RNase resistant fragments with a peak at 3*S* (Fig. 1a). However, if [^{3}H]-adenine labelled mRNP was digested with a higher concentration of RNase, then – as had been expected – a 9*S* RNase resistant peak characteristic of the polysomal poly(A)-protein particles of rat liver (Tomcsányi et al., 1976, 1981a) was obtained (Fig. 1b).

When [^{3}H]-adenine labelled mRNP was treated with RNase at a concentration of 0.1 μg/ml a 3*S* peak, similar to that obtained in the case of [^{3}H]-orotic acid labelled mRNP, and in addition a small 9*S* peak appeared (Fig. 1b). It seems that low concentration RNase failed to liberate all poly(A)-protein particles from mRNP. It should be noted that in the case of 0.1 μg/ml RNase treated mRNP about 20–25% of radioactivity applied to the gradients could be recovered from the 2–5*S* zone and about 50% of the material sedimented to the bottom of

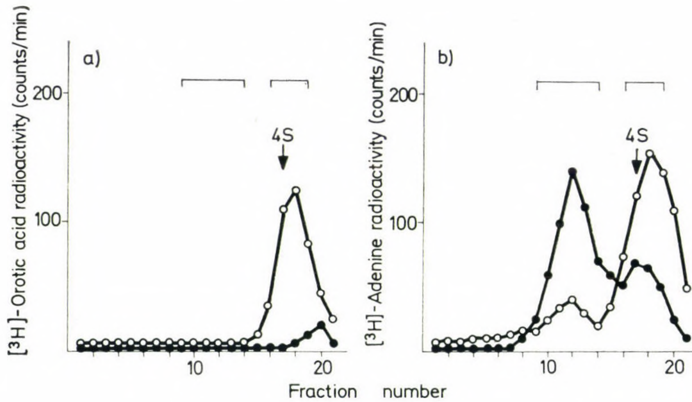


Fig. 1. Sucrose gradient centrifugation of RNase treated 12—21*S* mRNP. [^{3}H]-orotic acid and [^{3}H]-adenine labelled 12—21*S* mRNP was prepared as previously described (Tomcsányi et al., 1981b) and treated with 5 μg/ml or 0.1 μg/ml RNase A at 37 °C for 30 minutes. Samples of RNase treated mRNP were layered on 32 ml of 5—20% sucrose gradients in buffer A and centrifuged at 26 000 r.p.m. for 24 hours in a SW 27 rotor. The fractions indicated were pooled, precipitated with two volumes of ethanol and used for further analysis. (a) [^{3}H]-orotic acid labelled RNase treated mRNP. (b) [^{3}H]-adenine labelled RNase treated mRNP. ●—●, 5 μg/ml RNase; ○—○, 0.1 μg/ml RNase. The arrows indicate the position of rat liver tRNA centrifuged under identical conditions. Direction of sedimentation is from right to left

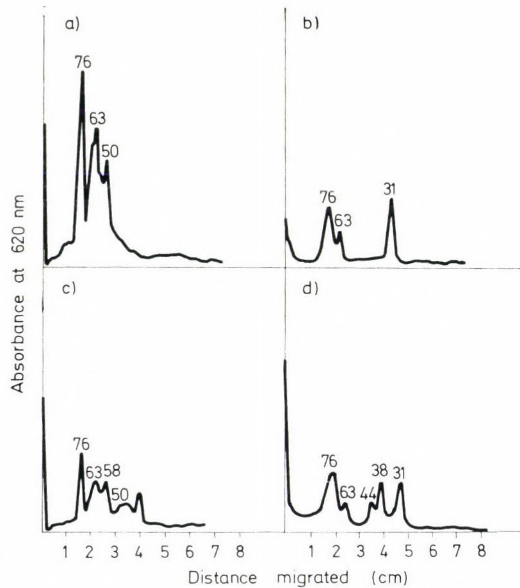


Fig. 2. Urea/SDS polyacrylamide gel electrophoresis of mRNP fragments. Alcohol precipitated samples from zones 7—12*S* and 2—5*S* were prepared for electrophoresis and electrophoresed in 8% polyacrylamide gels containing 0.1% SDS and 6 M urea. (a) Polypeptides in the 7—12*S* zone of 5 μg/ml RNase treated mRNP; (b) polypeptides in the 2—5*S* zone of 5 μg/ml RNase-treated mRNP; (c) polypeptides in the 7—12*S* zone of 0.1 μg/ml RNase treated mRNP; (d) polypeptides in the 2—5*S* zone of 0.1 μg/ml RNase treated mRNP

the tubes. Digestion of mRNP with 1 μg/ml RNase gave results similar to that of 5 μg/ml RNase (not shown).

The polypeptides present in the 7–12*S* and 2–5*S* zones of RNase treated mRNP are shown in Fig. 2. Six polypeptides exhibiting molecular weights of 50 000 (P50), 54 000 (P54), 58 000 (P58), 63 000 (P63), 76 000 (P76) and 90 000 (P90), typical of rat liver poly(A)-protein particles (Tomcsányi et al., 1981a), were found in the 7–12*S* zone, if the mRNP was digested with 5 μg/ml of RNase (Fig. 2a). In the 2–5*S* zone of 5 μg/ml RNase-treated mRNP there were three polypeptides, namely P31, P63 and P76 (Fig. 2b). The 7–12*S* zone of 0.1 μg/ml of RNase treated mRNP contained the polypeptides of poly(A)-protein particles and in addition a small amount of P31, P38 and P44 (Fig. 2c). When the polypeptides of the 2–5*S* zone of mRNP digested with low concentration RNase were analyzed, five polypeptides, namely P31, P38, P44, P63 and P76 were found (Fig. 2d). The polypeptide patterns of the appropriate zones of RNase treated mRNP, originated either from mRNP labelled with [^{3}H]-orotic acid or with [^{3}H]-adenine, were similar to each other.

The size distribution of RNA fragments derived from zones 7–12*S* and 2–5*S* of RNase treated mRNP was examined by polyacrylamide gel electrophoresis (Fig. 3). The 7–12*S* zone of [^{3}H]-adenine labelled mRNP contained polynucleotide fragments (Fig. 3a) resembling the poly(A) of poly(A)-protein particles in electrophoretic mobility (Tomcsányi et al., 1981a), while in the 2–5*S* zone

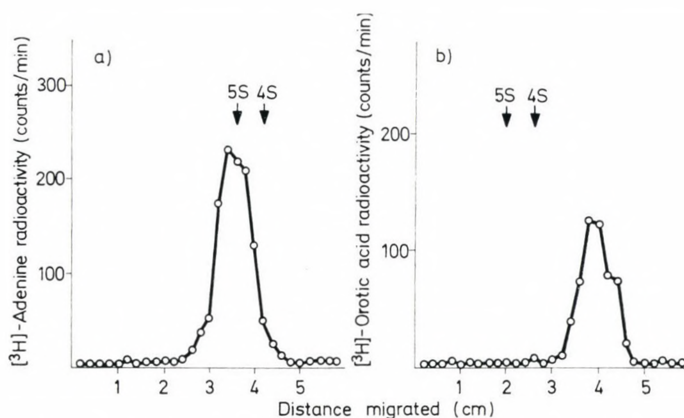
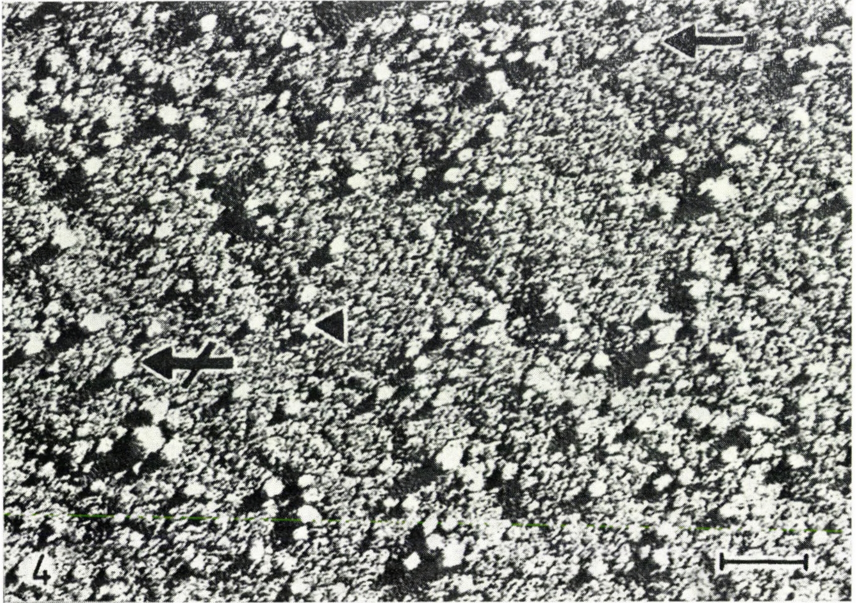
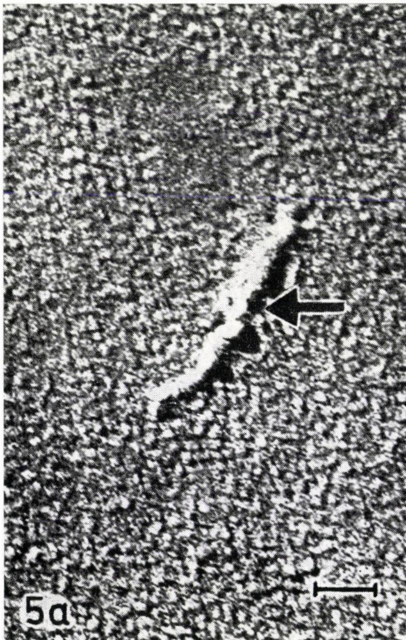


Fig. 3. Polyacrylamide gel electrophoresis of [^{3}H]-adenine and [^{3}H]-orotic acid labelled polynucleotide fragments from the 7–12*S* and 2–5*S* zones of RNase treated mRNP. RNA was prepared from the 7–12*S* and 2–5*S* zones of sucrose gradients by phenol-chloroform (Perry et al., 1972), precipitated in the presence of carrier rat liver tRNA, dissolved in 0.2% SDS, 2 mM EDTA, 20 mM NaH_2PO_4, 40 mM Tris-HCl (pH 7.4) 10% glycerol and heated at 75 °C for 5 minutes before application to the surface of 3% or 10% polyacrylamide gels prepared according to Hirsch and Penman (1974). (a) [^{3}H]-adenine labelled mRNP treated with 5 μg/ml RNase, 7–12*S* zone, 3% gel. (b) [^{3}H]-orotic acid labelled mRNP treated with 0.1 μg/ml RNase, 2–5*S* zone, 10% gel. Marker [^{14}-C]-labelled tRNA and 5*S* RNA were run in separate gels





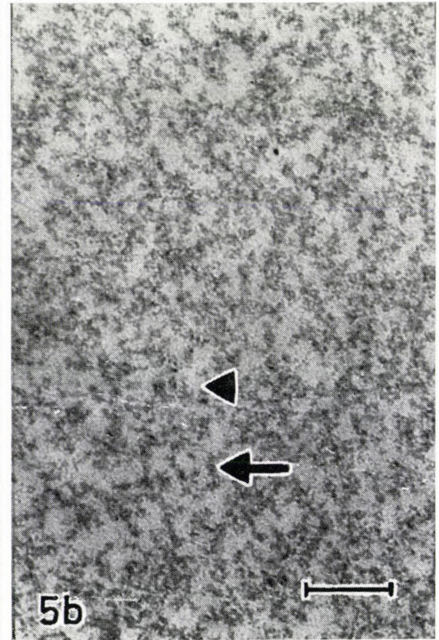


Fig. 4. Electron micrograph of 12−21*S* mRNP. Platinum-palladium shadowing. Particles differ in diameter: ◄⁄— 20.3 nm, ◄— 16.6 nm, ◄ 9.2 nm in diameter. Magnification ×60,000. The bar represents 200 nm

Fig. 5. Electron micrographs of 12−21*S* mRNP. (a) Fibril-like structure of mRNP. The arrow showsp oint of juncture between two fibril-like structures. Platinum-palladium shadowing. Magnification ×45,000. The bar represents 200 nm. (b) Ultrathin section of sedimented 12−21*S* mRNP. ◄ globular mRNP particles, ◄— fibril-like mRNP. Fixed in 2.5% glutaraldehyde and 1% OsO_4. Magnification ×120,000. The bar represents 100 nm

([^{3}H]-orotic acid labelled, 0.1 μg/ml RNase treated mRNP) there were components which migrated more rapidly than tRNA (Fig. 3b). These rapidly migrating components represented polynucleotide sequences consisting of approximately 16–50 residues with a maximum value of about 30 nucleotides.

In platinum-palladium shadowed preparations electron microscopy of partially fragmented mRNP liberated from polysomes by EDTA treatment showed single, globular particles (Fig. 4). The diameter of the particles was in the range of 7–21 nm in every fraction of the 12–21*S* zone. However, fibril-like structures of about 300–400 nm in length were also observed. These structures tended to aggregate and form end-to-end complexes as shown in Fig. 5a. Along the fibrils 6 to 7 blobs up to 10 nm in diameter could be seen. In ultrathin sections of sedimented mRNP globular and fibril-like structures, resembling those observed in shadowed preparations, were visible (Fig. 5b).

Discussion

In previous papers we have reported the protein composition of rat liver mRNP (Tomcsányi et al., 1981b) and of polysomal poly(A)-particles (Tomcsányi et al., 1981a). Since in mRNP there are more polypeptides (P29, P31, P38, P44, P50, P54, P58, P63, P78, P105 and P130) than in its poly(A)-protein particle (P50, P54, P58, P63, P76 and P90) the association of mRNA sequences with proteins other than the poly(A) segment was supposed. The result of the present investigation provides more direct evidence for this supposition. It is demonstrated that treatment of mRNP with high and low concentrations of pancreatic RNase produces different types of ribonucleoprotein particles. Digestion of mRNP with RNase at a concentration of 5 μg/ml resulted in particles which according to their characteristic sedimentation, electrophoretic mobility of the RNA moiety and protein composition are no doubt 9*S* polysomal poly(A)-protein particles of rat liver. On the contrary, low concentration RNase produced 2–5*S* (on an average 3*S*) ribonucleoprotein fragments contained RNA pieces which consisted of about 16–50 nucleotides. Together with these RNA fragments five polypeptides, P31, P38, P44, P63 and P76, were found. P63 and P76 proved to be poly(A)-bound polypeptides (Kish, Pederson, 1976, Jain et al., 1979; Tomcsányi et al., 1981a). The presence of these poly(A)-bound polypeptides in the 2–5*S* zone of the gradients can be explained by the findings in Brawerman's laboratory (Bergmann, Brawerman, 1977; Brawerman, 1979) according to which the poly(A)-protein particles become unstable after liberation from polysomes. We have observed that the resedimentation of rat liver 9*S* poly(A)-protein particles, depending on the method of concentration of the particles, gave more (Tomcsányi et al., 1976) or less (Tomcsányi et al., 1981a) [^{3}H]-adenine labelled material in the 2–5*S* zone of the gradient. In this zone poly(A) chains consisting of approximately 30–40 residues were found together with P63 and P76 (unpublished results). Therefore P63 and P76 derived from degradation of poly(A)-protein particles. However,

P38 and P44 must have been associated with non-poly(A) sequences of mRNA since they protected RNA pieces, labelled in the pyrimidines, against RNase. P31, which is certainly not a poly(A)-bound polypeptide, may have been associated with RNA sequences having low pyrimidine content and high RNase resistance since a low level of pyrimidine radioactivity was found after high RNase treatment.

Our finding, in regards to the resistant fragments, is in accordance with the results of others. Jeffery (1978) had treated oligo(dT)-cellulose bound Ehrlich ascites mRNP with pancreatic RNase and obtained 2–3*S* non-poly(A) RNP fragments. Goldenberg et al. (1979) reported that digestion of 15*S* duck globin mRNP with staphylococcal nuclease resulted in [^{3}H]-uridine labelled specific RNA fragments protected by proteins. Thus our results confirm that in animal cells other mRNA sequences than poly(A) are involved in RNA-protein interaction.

The electron microscopy of mRNP revealed two things: (a) Treatment of the polysomes with EDTA liberates the mRNP mainly as globular particles and only a small proportion of it remains in fibril-like form, which is probably the physiological appearance of mRNP. This observation is in accordance with that of Liautard (1977) who, on the basis of sucrose gradient electrophoresis, reported surface changes of HeLa mRNP caused by EDTA. (b) When mRNP fibrils were observed several blobs could be seen along their lengths. These blobs were probably proteins attached to mRNA. Therefore, the mRNA chain in the mRNP is not evenly coated with proteins but the proteins are clustered in packages at specific points along the mRNA. These latter findings are in agreement with those of Dubochet et al. (1973) who first visualized duck and rabbit globin mRNP particles by the electron microscope.

On the basis of the results reported here and in a previous paper (Tomcsányi, et al. 1981b) we suggest a hypothetical model for the "average" mRNP molecule in rat liver. The number average nucleotide length of the poly(A)-containing mRNA molecules from rat liver polysomes is estimated to be 1475, including a poly(A) segment composed of 133 adenylate residues (Sala–Trepat et al., 1978). The mRNA molecule having such a size exhibits a molecular weight of about 500 000 of which the participation of the poly(A) segment is about 47 000. Since the RNA : protein ratio in rat liver mRNP is about 1 : 2 (preceding paper), this gives a value of about 1 000 000 daltons of protein per mRNA. Polypeptides with a molecular weight of about 200 000 are capable of binding to poly(A) (Tomcsányi et al., 1981a), therefore the remaining 800 000 daltons of proteins must be attached to the non-poly(A) region of mRNA. The deviation of Coomassie brilliant blue staining of proteins from Beers law has been reported (Pederson, 1974; Cardelli, Pitot, 1977), therefore the following calculation of the number of polypeptides bound to an "average" molecule is only meant to be an approximation. If all non-poly(A) bound polypeptides were present in the mRNP, about two molecules of each could attach to the non-poly(A)-region of mRNA since the sum of their molecular weights is about 750 000. However, among the mRNP proteins, it is P38 that is found in the largest quantity. Assuming that the base-to-base distance

is 0.265 nm (Naora, Fry, 1977) the length of a mRNA chain is about 355 nm, without poly(A). Along a mRNP fragment (about 300 nm in length) 6 to 7 protein packages were observed, thus on a 355 nm long RNA chain 8 to 9 of it could be located. P38 being the most frequent of the mRNP proteins, it may be supposed that this polypeptide is present in all protein packages, therefore one can calculate that there are nine P38 present in an "average" mRNA molecule (43% of non-poly(A) bound proteins). On the basis of the available data it is difficult to estimate the actual number of other proteins bound to mRNA but it is probable that of some of them, e.g. P31 and P44, more than one molecule may occur. More work is needed to determine what mRNA sequences are involved in the binding of proteins and what role these proteins play in the various aspects of mRNA metabolism.

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The Mechanism of Limited Tryptic Proteolysis of Heavy Meromyosin as Revealed by Peptide Analysis

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To elucidate some ambiguous details in the tryptic fragmentation scheme of HMM as given by Bálint et al. (J. Biol. Chem. *250* (1975) 6168; Arch. Biochem. Biophys. *190* (1978)793), the peptide fragments were isolated by a milligram scale preparative gel electrophoresis procedure.

The dansyl-peptide map of the 20 kDal tryptic fragment obtained fromt tryptic heavy meromyosin (HMM) and that of a similar fragment from papainic subfragment-1 (S-1) were found to be nearly identical. This finding gives unequivocal proof for the location of the 17 kDal peptide stretch, lost during digestion in the form of small peptides, at the C terminal part of the heavy chain backbone of HMM.

The N terminals of the 150, 74, and 25 kDal fragments of the heavy chain isolated from HMM digested by trypsin under widely differing conditions were shown to be acetylated. The N terminal amino group of the other peptide fragments of HMM remains the same under widely differing conditions of digestion. We conclude that all the fragments are well defined polypeptides and digestion progresses by splitting from the C terminals formed by the primary splits.

Introduction

The proteolytic susceptibility of the heavy chain of the myosin molecule in the native state is quite limited. Of the 380 Arg + Lys residues only a few are accessible to trypsin at low enzyme/substrate ratio. The few splits that occur give rise to the production of a few definite polypeptide fragments which remain associated up to a very late state of digestion due to secondary and tertiary interactions.

The "family tree" of the resulting fragments (Fig. 1a) could be elucidated from their patterns in analytical $NaDodSO_4$ gels during digestion (Bálint et al., 1975a).

A sequential localization of these polypeptide species was suggested in a subsequent paper (Bálint et al., 1978). This scheme (3B, in Fig. 1b) was generally accepted in spite of some undecided details (Yamamoto, Sekine, 1979; Mornet et al., 1979). The most conspicuous uncertainty concerns the location of the 17 kDal piece lost (presumably digested to small peptides) during the transformation of

Abbrevations: HMM, heavy meromyosin; S-1, subfragment 1; S-2, subfragment 2; $NaDodSO_4$, sodium dodecyl sulfate; Dansyl, 1-dimethylamino-naphthaline-5-sulphonyl.

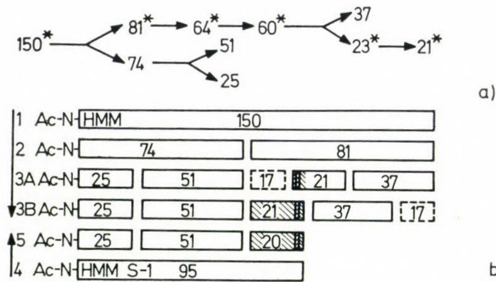

Fig. 1. Scheme of the tryptic fragmentation of the heavy chain backbone of HMM. a. "Family tree" of the polypeptide fragments formed during the prolonged limited tryptic proteolysis of the heavy chain of HMM as deduced from the time progress of the $NaDodSO_4$ electrophoresis pattern of HMM in the course of digestion. Numbers refer to the molecular weights of the polypeptide fragments in kDal determined by $NaDodSO_4$ polyacrylamide gel electophoresis. The asterisks indicate the location of the radioactivity attached to the SH-1 and SH-2 groups (according to Bálint et al., 1975 a., 1978). b. Sequential localization in the parent polypeptide chain of the main fragments referred to in "a". The arrows symbolize the progress of proteolysis by trypsin. Arrow pointing downwards: proteolysis of HMM, arrow pointing upwards: proteolysis of papain-produced S-1. 3A and 3B show the two final states equally possible on the basis of analytical $NaDodSO_4$ gel patterns as a function of time of protolysis. The densely hatched areas in the approximately 20 kDal fragments represent the decapeptide (Elzinga, Collins, 1977) separating the SH-1 and SH-2 sulfhydryls contained both in the 21 kDal HMM and 20 kDal S-1 fragments (Bálint et al., 1978). The lightly hatched areas represent the presumably identical stretches of the 21 kDal HMM and 20 kDal S-1 fragments

the 81 kDal peptide to the 64 kDal one. This ambiguity is shown in Fig. 1b by two equally possible versions (3A and 3B).

Additional unclarified problems of the fragmentation scheme arise from the limited resolution of the analytical $NaDodSO_4$ electrophoresis: it is uncertain whether these fragments of the heavy chain are well-defined stretches in terms of sequence analysis or whether they are groups of polypeptides differing at the ends by small pieces, the differences in molecular weight being too small to be detected.

To elucidate these details, we have worked out an effective, milligram scale preparative gel electrophoretic method for the isolation of the different polypeptide fragments. This made it possible to compare the peptide maps of some relevant fragments, to find the fragments blocked at the N terminus (c.f. Starr, Offer, 1973), as well as to show that the blocking group is acetyl. It could also be shown that the unblocked peptides have well-defined N terminals which remain the same for the respective fragments independently of the widely differing conditions of digestion used.

Materials and methods

Myosin, tryptic HMM and HMM S-1 (obtained by papain digestion of myosin) were prepared as previously described (Bálint et al., 1975a, b). Further digestion of HMM-S-1 by trypsin was carried out as described by Bálint et al.,

(1978). The tryptic digestions were done with "Type III Sigma" trypsin. Digestion was stopped by addition of $NaDodSO_4$ at a final concentration of 1%, immediately followed by boiling for two minutes. Carboxymethylation was carried out according to Elzinga (1970) with the modification that 1% $NaDodSO_4$ was used instead of urea. The carboxymethylated material was dialyzed exhaustively against distilled water.

Dansylation was carried out according to Kinoshita et al., (1974) but again 1% $NaDodSO_4$ was used as denaturant instead of urea. The dansylated material was exhaustively dialyzed against 10 mM NH_3 and lyophilized.

The carboxymethylated and dansylated proteins were subjected to preparative electrophoresis on $NaDodSO_4$ polyacrylamide gels. The equipment was constructed in our workshop essentially on the principle of simple analytical equipments. The scaled-up apparatus was able to accept 12 gel columns each 25 mm in diameter and 160 mm in length. To keep the pH and ionic strength constant in this scaled-up apparatus a pump was required to ensure proper circulation of the 8 l electrode buffer.

The gel was 8% for total acrylamide, 2.7% of which was bis-acrylamide. Gel and running buffers contained 0.03% $NaDodSO_4$ and 0.05 M Tris-maleate, pH 7.0 (Tris base was adjusted with maleic acid to the desired pH). 0.7 mg protein was applied per cm^2 and the current density was 3 mA/cm^2. Separation took 15 hours electrophoresis at room temperature. Dansylated protein bands were identified under long wave length (360 nm) ultraviolet light, cut out, ground and extracted three times for 2 hours with 3–5 volumes of a solution containing 0.1% $NaDodSO_4$, 0.01 M sodium phosphate buffer, pH 7.4 at 37 °C. The extracts were then exhaustively dialyzed against distilled water and lyophilized. In one equipment cca. 40 mg protein mixture can be separated with 50% yield. The procedure takes 3 days. The effectiveness of the separation can be seen in Fig. 2.

For further processing the lyophilized material was dissolved in the above buffer and insoluble material removed by centrifugation at 5000 *g*. Protein was precipitated by 9 volumes of acetone, the precipitate centrifuged and washed, first with 80% and then with 100% acetone. The acetone washed material was used for N terminal analysis.

Dansylated fragments were hydrolyzed for 4–6 hours at 105° by 6 N HCl in N_2 atmosphere (Gros, Labouesse 1969). The hydrolyzate was dried in vacuo over P_2O_5 and KOH. The dansyl-amino acids were extracted with water saturated ethyl acetate. The extract was evaporated in N_2 stream and the dansyl amino acids were dissolved in a solvent containing 90% ethanol and 10% 1 M NH_3. Dansyl amino acids corresponding to 1–2 nmol protein were separated by thin-layer chromatography on 5×5 cm polyamide sheets according to Woods and Wang (1967).

For the identification of the putative acetylated N terminal residue of the 150 kDal, 74 kDal, and 25 kDal fragments we used the hydrazinolysis-dansyl method of Schmer and Kreil (1969) with the following modification: 1-acetyl-2-dansyl-hydrazine was identified on polyamide thin-layer rather than on silica gel

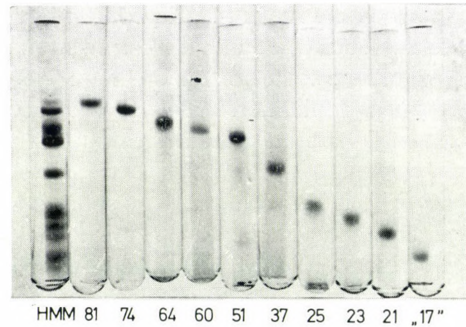

Fig. 2. Illustration of the electrophoretic purity of the fractions obtained by preparative gel electrophoresis as described in "Methods". HMM was digested by trypsin in the presence of 3 mM EDTA for 5 min and then separated to fractions in the preparative system. The first gel column shows the analytical picture of the digested HMM. The following gels show samples of the isolated fractions in the order of decreasing molecular weight. Gels were loaded by about 40 μg unfractionated or 10 μg isolated protein. The smallest molecular weight fraction showing up as an apparently 17 kDal fragment in beyond the resolving power of the separation as shown by the heterogeneity of N terminals (Table 2)

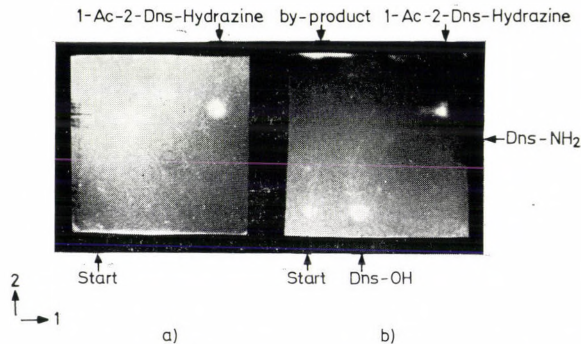

Fig. 3. Demonstration of the presence of acetyl residue in the 25 kDal fragment by hydrazinolysis. The dansyl derivatives extracted from the hydrazinolysates of about 2 nmol protein by chloroform were chromatographed on polyamide thin-layer sheets in the following solvent systems: 1.5% formic acid (1) and benzene–acetic acid 9 : 1 (2). The chromatogram was photographed in UV. light. a: Reference, b: 25 kDal fragment of HMM

by the solvent systems of Woods and Wang (1967) developed for dansyl amino acids.

Comparison of the 20 kDal tryptic fragment of S-1 and the 21 kDal tryptic fragment of HMM was done with fragments without dansylation. These fragments were detected in the preparative gel according to Nelles and Bamburg (1976) and isolated by the same method described for dansyl derivatives. Peptide maps were obtained by tryptic digestion according to Elzinga (1970) (trypsin to protein ratio:

1 to 20 (w/w), 20 °C, 20 hrs). For these experiments "Type XI" trypsin of Sigma was used. Dansylation of the digest was carried out according to Tamura et al., (1973). Dansyl peptides were purified on Dowex 50 × 2 H^+ columns (10 × 0.5 cm) according to Schmer and Kreil (1967) followed by two dimensional chromatography on polyamide sheets (15 × 15 cm) according to Gerday et al. (1968). 2 – 5 nmol protein was used for the preparation of one peptide map. Development in the first dimension was done with a mixture of formic acid: H_2O = 3 : 200 (vol : vol) and in the second dimension with xylene : pyridine : acetic acid = 10 : 1 : 1 (vol : vol).

Amino acid analysis from about 2 – 2 mg protein was carried out on a Chinoin OE 1975 instrument after hydrolysis for 24 hours at 105° in 6 N HCl in vacuo.

Analytical gel electrophoresis was carried out according to Weber and Osborn (1969) in a 6% gel, with bis acrylamide being 2.7% of the total acrylamide present.

Protein concentrations were determined according to Lowry et al. (1951).

Results and discussion

It was found by Bálint et al. (1978) that the two enzymatically essential thiol groups (SH-1 and SH-2) are equally present in both HMM and S-1, even after prolonged digestion. Lu et al. (1978) found the same for the unique methylhistidine residue. This suggests that these two fragments (HMM and S-1) contain within them the same section of the heavy chain of myosin. The sequence work of Yamashita et al. (1974) and of Elzinga and Collins (1977) have shown, however, that these three groups are relatively near each other. They span only a 6.75 kDal peptide

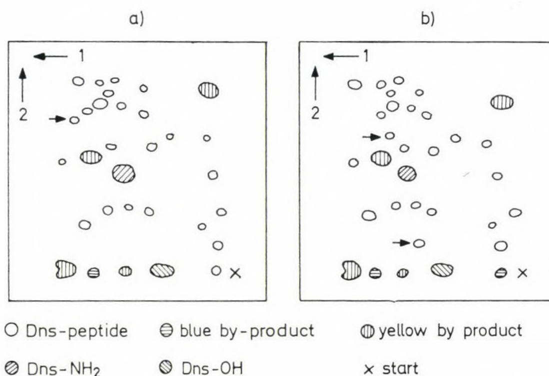

Fig. 4. Dansyl-peptide map of the tryptic digest of the 21 kDal HMM fragment and that of the 20 kDal fragment of S-1. For each peptide map about 5 nmol peptides were used. Chromatography was performed on polyamide thin-layer sheets, for elution formic acid–water 3 : 200 (1) and xylene–pyridine–acetic acid 10 : 1 : 1 (2) were used. a: 21 kDal fragment of HMM, b: 20 kDal fragment of S-1

stretch. The location of the 17 kDal peptide segment depends critically on how far this common sequence reaches in the N terminimal direction: if it reaches somewhere near the C terminal of the 51 kDal fragment there does not remain enough place to locate the 17 kDal "gap" here (compare versions 3A and 3B in Fig. 1b). This also means that the approximately 20 kDal fragments of HMM and S-1 should be very nearly identical (compare 3B and 5 in Fig. 1b).

Amino acid analysis (Table 1) and peptide maps (Fig. 4) show that the 21 kDal polypeptide derived from HMM and the 20 kDal polypeptide derived from

Table 1

Amino acid composition of the 21 kDal HMM and the 20 kDal S-1 fragments isolated by preparative gel electrophoresis

The values reported are the means of two independent analyses, carried out on two different preparations. The difference index ("D. I.") was calculated according to Wallace and Dieckert (1976). An index of 100 means completely different composition, an index of zero full identity.

Amino acid	21 kDal mol%	20 kDal mol %	Δ mol%
Lys	8.42	7.41	1.01
His	2.36	1.93	0.43
Arg	6.60	4.79	1.81
Asp	8.58	7.56	1.02
Thr	5.09	6.10	1.01
Ser	5.24	6.38	1.14
Glu	13.36	14.12	0.76
Pro	5.48	5.32	0.16
Gly	9.26	9.19	0.07
Ala	6.60	7.79	1.19
Cys	1.37	1.33	0.04
Val	3.34	5.13	1.79
Met	0.91	0.97	0.06
Ile	5.76	6.72	0.96
Leu	10.32	8.53	1.79
Tyr	1.75	1.57	0.18
Phe	5.54	5.13	0.41
	100	100	D. I. = 6.92

S-1 are nearly identical. This places the 17 kDal piece lost during digestion of HMM definitely at the C terminal of the heavy chain backbone of HMM (version 3B in Fig. 1b).

The results of N terminal determinations of fragments isolated from HMM samples digested under different conditions are summarized in Table 2. It can be seen that tryptic fragments derived from HMM are well-defined peptides (at least as far as the N terminus is concerned). Increasing the time of digestion or adding

Table 2

Results of the N terminal determinations carried out on different peptides isolated by preparative gel electrophoresis from HMM digested under different conditions as indicated

Strongly fluorescent spots were considered as N terminal residues while weakly fluorescent spots were regarded as reproducible side products

	150 kDal	81 kDal	74 kDal	64 kDal	60 kDal	51 kDal	37 kDal	25 kDal	23 kDal	21 kDal	17 kDal
HMM*	Ac-N-	Gly Lys	Ac-N-	Gly Lys	Gly Lys	Met (Lys)					Leu Ala, Glu Gly, Lys
HMM digest. 5 min 3 mM EDTA		Gly Lys		Gly Lys	Gly Lys	Met (Lys)	Lys		Gly Lys	Gly Lys (Glu)	Leu Ala, Glu Gly, Lys
HMM digest. 15 min 3 mM EDTA						Met (Lys)	Lys	Ac-N	Gly Lys	Gly Lys (Glu)	Leu Ala, Glu Gly, Lys
HMM digest. 30 min 3 mM EDTA						Met (Lys)	Lys (Ala)		Gly Lys	Gly Lys (Glu)	Leu Ala, Glu Gly, Lys
HMM digest. 45 min 3 mM EDTA						Met (Lys)	Lys (Ala)		Gly Lys	Gly Lys (Glu)	Leu Ala, Glu Gly, Lys
HMM digest. 5 min 3 mM Ca^{2+}		Gly Lys		Gly Lys		Met (Lys)			Gly Lys		Leu Ala, Glu Gly, Lys

* Note that even an HMM prepared by a very short tryptic digestion contains a number of more or less jagged molecules. (Bálint et al., 1975a).

or withdrawing of Ca^{2+} ions, does not influence the nature of N terminal residues. The significance of the finding of such well defined N terminal amino acid(s) in all fragments is underlined by the fact that the apparently 17 kDal protein fraction obtained upon preparative gel electrophoresis gave at least five spots fluorescing with equal intensity. This fraction seems to represent debris of unknown origin (and has of course no relation to the 17 kDal piece lost from the heavy chain in the course of digestion).

The identification of the N terminus of the 37 kDal fragment as Lys agrees with the finding of Lu (1980) who published the sequence of the first seventeen amino acids (from the N terminus) of this fragment.

There were no N terminal groups identifiable by the dansyl method in the 150 kDal, 74 kDal and 25 kDal fragments. The presence of an acetylated residue in all these fragments was shown by hydrazinolysis as described in "Methods". Figure 3, showing the presence of 1-acetyl-2-dansyl-hidrazin in the 25 kDal fragment of HMM subjected to hydrazinolysis serves as an example.

These findings are in agreement with the finding of Starr and Offer (1973) on the acetylated nature of the N terminus of the myosin heavy chain and settle experimentally the assumption of Bálint et al. (1975a) placing the N-terminus of the heavy chain at the N-terminus of the 25 kDal fragment. This finding is also consistent with the result of sequence studies of Lu et al. (1978) on the 25 kDal tryptic fragment. The N terminal data summarized in Table 2 also suggest that the 64 kDal → 60 kDal and the 23 kDal → 21 kDal steps (see Fig. 1a) are results of proteolysis proceeding from the respective C terminals since the N terminals remain unchanged. Strictly speaking, however, the rather faint possibility of sequential splitting from the N terminus giving repeatedly the same N terminals is not excluded by these results.

The double nature of the N terminals calls for some comments: in most cases we obtained two fluorescent spots of practically equal visual intensity. If Lys is the N terminus, it is present (of course) as bis-dansyl-Lys with approximately twice the fluorescence intensity of monodansyl amino acids. We may therefore conclude that in these fractions the predominant N terminal amino acid is Gly, with a substantial fraction of the polypeptides having a Lys N terminal. A quite plausible explanation would be that these fragments originate from the random tryptic splitting of a Lys-Lys-Gly of Arg-Lys-Gly sequence. Once Lys becomes, as a result of this splitting, an N terminal amino acid, it becomes rather resistant to attack by trypsin which is essentially an endopeptidase. However, the possibility that enzymatic hydrolysis occurs in two regions separated only by a few amino acids cannot be excluded.

We hope that the details of the fragmentation scheme reported in this paper will be of serious value for the determination of the primary sequence of the heavy chain of myosin going on now in several laboratories.

We are indebted to Professor John Gergely (Boston) for his valuable comments on this work during preparation of this manuscript.

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Studies on Human Tonsillar Lymphocyte Membrane

III. Isolation and Characterization of Plasma Membrane Fractions after Hypotonic Lysis

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Hypotonic lysis was applied for the preparation of lymphocyte plasma membranes. By this method it was possible to separate membrane fractions with a high sialic acid to protein and cholesterol to phospholipid ratio. The plasma membrane fractions had a lower content of endoplasmatic reticulum than the preparations described earlier but were slightly contaminated with DNA.

The isolation method proved to be useful for the preparation of surface membranes from human lymphocytes.

Introduction

Isolation of thoroughly pure membraneous fractions from eukaryotic nucleated cells is a procedure that has not been developed as yet. The crucial point of the isolation is the disruption of cells. It can be performed by hypotonic lysis (Emmelot, Bos, 1969; Ray, 1970; Wong, Zull, 1974), by isotonic homogenization (Emmelot et al., 1964; Allan, Crumpton, 1970, 1972; Demus, 1973; Kornfeld, Siemers, 1974; Hrabák et al., 1977), by nitrogen cavitation (Ferber et al., 1972; van Blitterswijk et al., 1973), etc. But each method has its drawbacks. Attempts to find a suitable procedure to eliminate contaminations were not successful.

Plasma membranes from lymphocytes have usually been prepared by isotonic homogenization (Allan, Crumpton, 1970; Demus, 1973; Hrabák et al., 1977). Membrane preparations of different compositions were isolated from tonsillar lymphocytes, spleen or lymph node cells, peripheral blood lymphocytes and thymocytes. The isotonic method has the advantage of facilitating the separation of cellular components and structures (mitochondria, lysosomes, intracellular membranes) in intact state. By this procedure small vesicles and small membraneous fragments associated with the microsomal fraction can be obtained from the plasma membranes. The plasma membrane particles may hardly be separated from other membraneous structures involved in the microsomal fraction. The main contaminations in the preparations are generally the fragments of the smooth ER(SER).

Abbreviations: ER, endoplasmatic reticulum; TCA, trichloroacetic acid.

Our purpose was to obtain plasma membranes relatively free of intracellular membrane fragments. In order to avoid separation problems mentioned above, hypotonic lysis was applied for disruption of the cells. By this method larger vesicular structures are formed from the plasma membranes and pelleted together with the nuclei (Emmelot, Bos, 1969; Ray, 1970; Jett et al., 1977). After pelleting the nuclear fraction the membraneous vesicles can be separated by density gradient ultracentrifugation. This method favours the isolation of purified plasma membranes but it is not suitable for the preparation of intact mitochondria.

Hypotonic lysis yielded good results in the preparation of liver (Ray, 1970; Wong, Zull, 1974) and kidney (Malbon, Zull, 1974) cell membranes, and in the case of mammalian erythrocytes as well (Dodge et al., 1963). Our experiments were based upon these experiences.

Materials and methods

Cells

Human lymphocytes were isolated from tonsils of children (3 – 10 years) by the method of Antoni and Staub (1978) and washed three times with Hanks' medium at 4 °C. 15 – 20 tonsils were used for obtaining $2 \times 10^9 - 10^{10}$ cells.

Preparation of plasma membranes (Fig. 1)

Washed cells were suspended in a hypotonic disrupting medium composed of a hundredfold diluted Hanks' solution containing 7.5 mM $MgCl_2$ then incubated at 4 °C for 120 min. Nuclear fraction was pelleted at 1000 *g* (Pellet I). The supernatant was centrifuged at 48 000 *g* to remove intracellular and small plasma membrane particles (Pellet I). Pellet I was washed three times with 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM $CaCl_2$ to eliminate the excess Mg^{2+}, it was then suspended in 3 ml of 50% (w/v) sucrose solution and layered on 3 ml of 60% (w/v) sucrose solution. 3 ml sucrose solutions (30% and 40%) were subsequently layered on the suspension. In each fraction sucrose was dissolved in 10 mM Tris-HCl buffer (pH 7.4). Pellet I was centrifuged for three hours at 38 000 rpm (rotor SW 41) in a Spinco L2-65 B Beckman ultracentrifuge. Interphases were sucked off, washed three times with Tris-HCl buffer (10 mM, pH 7.4), resuspended in the same medium and stored at −20 °C.

Chemical determinations

Protein content was determined by the method of Lowry et al., (1951). DNA and RNA were separated on the basis of the method worked out by Fleck and Munro (1962). DNA was measured according to Burton (1956); RNA was

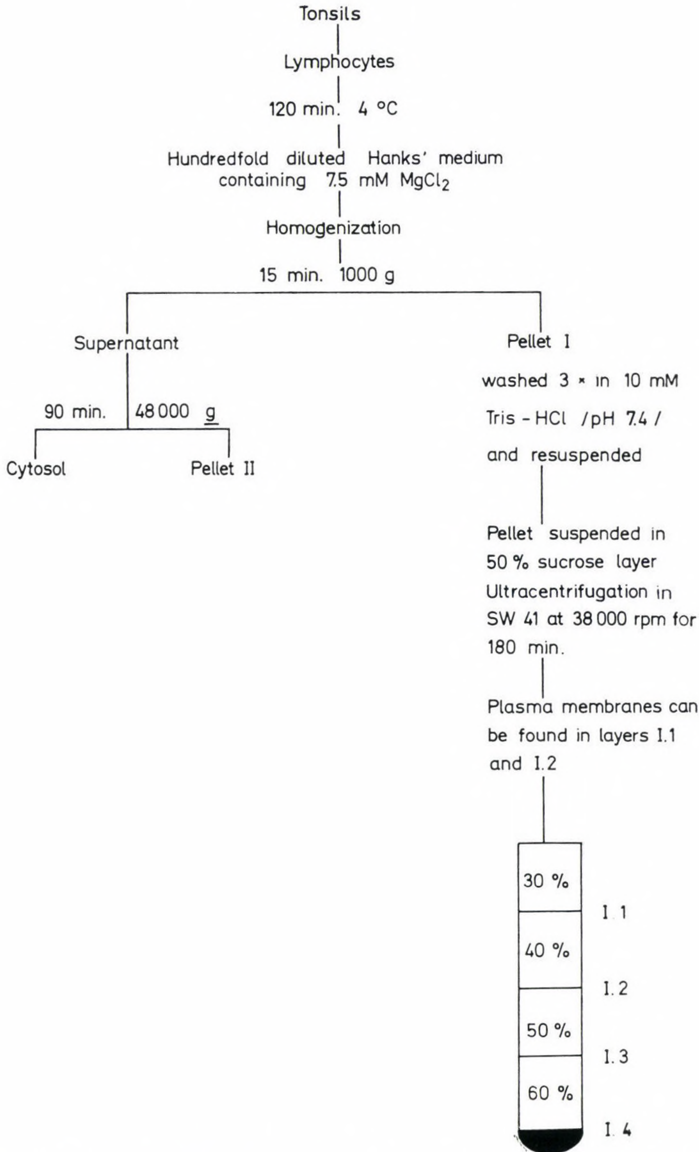

Fig. 1. Scheme of the preparation of plasma membranes from human tonsillar lymphocytes

determined by the orcinol reagent (Schneider, 1957). For the determination of sialic acid (Warren, 1959) samples were hydrolyzed in 0.1 N H_2SO_4 for 1 hour. To determine cholesterol and phospholipids, lipids were extracted twice with chloroform–methanol (2 : 1), then the samples were divided in two parts. Cholesterol was determined by acetic anhydride reagent (Stadtman, 1957). In order to estimate

phospholipids, samples were dried and hydrolyzed in 1 ml of 70% $HClO_4$ (15 min, 150 °C), lipid phosphorus was determined by a slightly modified method of Chen et al., (1956).

Assay of enzymes

5'-nucleotidase was determined by the method of Heppel and Hilmoe (1955). Glucose-6-phosphatase was measured on the basis of inorganic phosphate release (Swanson, 1955). Acidic phosphatase was determined at pH 5.6; alkaline phosphatase at pH 8.8; 5'-adenosine monophosphate, glucose-6-phosphate and Na-β-glycerophosphate were applied as substrates. Released inorganic phosphate was determined as mentioned earlier in the supernatants of the TCA precipitates (5%).

Table 1

Protein distribution in subcellular fractions

The amount of protein is referred to 2×10^9 cells

Fraction	mg protein	% of total
Total protein (homogenate)	79.92±5.02	100
Pellet I	39.04±2.89	48.8
Pellet II	5.08±0.79	6.36
Cytosol	31.62±3.94	39.6
I.1	0.184±0.02	0.23
I.2	0.925±0.07	1.16
I.3	0.96±0.07	1.2
I.4	34.20±1.96	42.8
Protein recovered (pellets I+II+cytosol)	75.74±6.67	94.8

± S.E.M. was calculated from 3 experiments

Results and discussion

Protein distribution in the subcellular fractions is presented in Table 1. Cytosol and nuclei (I.4) represent more than 80% of the total cellular protein. I.1 + I.2 fractions (plasma membrane-rich fractions) contain 1.4% of the total protein.

Tables 2 and 3 show the enzymatic markers and the chemical compositions of the preparations. Analytical data seem to indicate that plasma membranes are accumulated in fractions I.1 and I.2. Specific activites of two enzymes, 5'-nucleotidase and alkaline phosphatase, considered as plasma membrane markers (Wallach, Winzler, 1974) were found to be the highest in subfractions I.1 and I.2, recoveries, however, were relatively low. Subfractions I.1 and I.2 contained 13% of

Table 2

Enzymatic markers in the subcellular fractions

Fraction	5′Nucleotidase		Glucose-6-phosphatase		Acidic phosphatase pH 5.6		Alkaline phosphatase pH 8.8	
	sp. activity	%	sp. activity	%	sp. activity	%	sp. activity	%
Homogenate	15.5±1.02	100.00	2.58±0.31	100.00	8.11±0.56	100.00	2.43±0.32	100.00
Pellet I	17.0±2.12	53.6	4.26±0.39	80.6	10.68±1.12	64.3	3.06±0.29	61.5
Pellet II	14.5±0.93	6.0	4.70±0.38	11.6	4.46±0.34	3.5	1.21±0.09	3.2
Cytosol	8.33±1.0	21.2	1.18±0.10	18.1	8.75±1.32	42.7	1.03±0.08	16.8
I.1	78.16±4.22	1.2 (2.2)	5.06±0.44	0.45 (0.56)	28.90±1.99	0.82 (1.28)	6.55±0.46	0.62 (1.01)
I.2	154.2±9.23	11.6 (21.6)	6.00±0.45	2.69 (3.34)	22.60±2.01	3.23 (5.01)	6.66±0.42	3.17 (5.16)
I.3	27.16±1.49	2.1 (3.9)	8.78±0.32	4.09 (5.07)	73.7±4.10	10.92 (26.2)	4.33±0.31	2.14 (3.48)
I.4	12.80±0.91	35.3 (65.9)	3.48±0.45	57.72 (71.57)	7.80±0.66	41.16 (63.9)	2.45±0.11	43.40 (70.14)
Recovery (pellets I+II+ +cytosol)		80.8		110.3		110.5		81.5
Recovery I.1–I.4		50.2 (93.6)		64.95 (80.53)		56.13 (96.4)		49.33 (79.8)

± S.E.M. was calculated from 4 experiments

Recovery % values in parentheses refer to Pellet I (100%)

Specific activities are given in nmol P_i liberated/mg protein/min

Table 3

Chemical composition

Fraction	DNA		RNA	
	μg/mg protein	%	μg/mg protein	%
Homogenate	189.0±22.0	100.00	32.3±2.7	100.00
Pellet I	196.0±15.0	56.7	43.1±4.9	65.2
Pellet II	39.0±4.0	1.3	36.4±2.9	7.2
Cytosol	11.0±1.6	2.3	18.7±3.3	22.9
I.1	16.1±3.0	0.02 (0.04)	36.4±3.0	0.26 (0.40)
I.2	23.2±1.6	0.14 (0.28)	40.3±5.6	1.44 (2.22)
I.3	163.6±19.5	1.04 (2.05)	53.9±4.0	2.0 (3.07)
I.4	229.6±21.2	51.98 (93.12)	47.2±6.1	62.5 (95.9)
Recovery (pellets I+II+cytosol)		60.3		95.3
Recovery I.1−I.4		53.18 (95.49)		66.2 (101.6)

± S.E.M. was calculated from 4 experiments
Recovery % values in parentheses are referred to Pellet I (100%)

the total (16% of the recovered) 5'-nucleotidase and 4% of the total (4.9% of the recovered) alkaline phosphatase activity.

The plasma membrane character of subfractions I.1 and I.2 was also supported by their increased sialic acid, cholesterol and P-lipid contents expressed as μg/mg protein. The molar ratio of cholesterol to phospholipid was lower than in some other plasma membrane preparations (Table 4), this may be attributed to the methods applied in the determination (Johnson, Robinson, 1979).

Our analytical results show that plasma membrane-rich subfractions are slightly contaminated by other subcellular components. Subfractions I.1 and I.2 seem to contain SER and lysosomal contaminations because glucose-6-phosphatase (characteristic of SER) and acidic phosphatase (marker of lysosome and lysosome-contaminated crude mitochondrial fractions) were also present in these fractions. However, SER contamination in our plasma membrane-rich fractions is much lower than in others (Table 4). The rough ER content (Table 4) is about the same as in other preparations, its presence is supported by the amount of RNA measured in subfractions I.1 and I.2

Subfractions I.1 and I.2 contain fairly much DNA. During isolation – in order to avoid larger DNA contamination – the intact state of nuclei must be

of subcellular fractions

Sialic acid		Cholesterol		Phospholipid	
μg/mg protein	%	μg/mg protein	%	μg/mg protein	%
32.7±5.60	100.00	90.5±8.8	100.00	227±22	100.00
11.2±1.9	16.8	61.8±5.2	33.4	151±8	32.5
51.2±6.7	10.0	65.8±2.3	4.6	105±12	2.9
15.0±1.9	18.2	146.0±26.0	63.8	274±36	47.8
65.5±8.8	0.46 (2.76)	270.0±34.0	0.69 (2.06)	866±97	0.88 (2.70)
27.4±5.3	1.01 (6.02)	178.1±21.1	2.36 (7.09)	834±104	4.25 (13.1)
1.1±0.2	0.04 (0.24)	102.0±14.2	1.35 (4.06)	68±8	0.36 (1.1)
0.2±0.04	0.39 (2.35)	53.3±3.2	25.2 (75.5)	54±5	10.18 (31.3)
	45.0		101.8		83.2
	1.9 (11.37)		29.6 (88.8)		15.67 (48.2)

maintained. 7.5 mM $MgCl_2$ was used for this purpose (Fig. 1), because it was found to be optimal for the preservation of nuclei on the basis of the measured DNA release (unpublished observations). Even so the DNA level was relatively high in such fractions I.1 and I.2. It is very likely that DNA contamination is due to the preparation method, since the plasma membrane-rich fractions were derived from the nuclear pellet. In lymphocytes the major part of cell volume is occupied by the nucleus and the surface membrane represents only a small portion of the nuclear pellet. Nevertheless, some authors believe that plasma membrane has its own DNA and it is not merely contamination (Lerner et al., 1971; Hall et al., 1971.)

We applied hypotonic lysis (diluted Hanks' medium) and a loose homogenization for disruption of the cells. Composition of the solution seems to be very important, i.e. in hypotonic Tris-HCl buffer or in isotonic NaCl solution cells tend to aggregate in concentrated suspensions. Phosphate, hydrocarbonate and – above all – Ca^{2+} ions play an important role in the prevention of cell clumping.

We think that our plasma membrane-rich subfractions (I.1 and I.2) from human lymphocytes are suitablepre parations for the purpose of biological experiments. Summarizing the advantages of the present method: 1) mild conditions for cell disruption (membranes are not broken to small fragments because of loose

Table 4

Comparison of the markers in different plasma membrane preparations

Membrane source	5'nucleotidase	Glucose-6-phospatase	Acidic phosphatase (pH 6)	Sialic acid	DNA	RNA	Cholesterol-P-lipid ratio mol/mol	Protein % of total
	nmol P/mg protein.min			μg/mg protein				
Human tonsils (1)	300.6	16.91	21.10	N. D.	9.0	17.5	0.72	0.91
Human tonsils (2)	141.8	34.06	17.16^{m}	17.8^{m}	3.3	39.16	0.75^{m}	0.80
Human tonsils (3)	116.3	5.53	25.75	51.4	19.6	38.3	0.53	1.39
Human thymocyte (4)	65.0	0.33	3.66	N. D.	0	25.0	0.75	3.4–13.6°/10 g
Calf thymocyte (5)	42.83	9.33	174.0^{p}	18.75	0.2	30.0	0.61	4.0
Calf thymocyte (6)	42.33	N. D.	N. D.	36.8	6.4	N. D.	N. D.	1.7
Pig lymph nodes (7)	168.3	5.00	8.50	11.0	0	28.0	1.01	1.31
Pig lymph nodes (8)	125.4	N. D.	N. D.	N. D.	70.2^{m}	36.5^{m}	0.53	1.27
Liver cells, rat (9)	223.0	23.3	93.3	9.4^{+}	N. D.	N. D.	0.38^{+}	N. D.
Rat thymocyte (10, 11)	137.3	2.85	13.8	N. D.	5.0	24.9	0.91	1.3

Results were calculated from the average of the different plasma membrane subfractions if they had been isolated from each other

^{m}= microsomal fraction; p = p-nitrophenyl-phosphatase; ° = mg protein/wet weight; $^{+}$ = Emmelot et al. (1964); (1) = Demus. 1973; (2) = Hrabák et al., 1977; (3) = this publication; (4) = Allan and Crumpton, 1972; (5) = van Blitterswijk et al., 1973; (6) = Kornfeld and Siemers, 1974; (7) = Allan and Crumpton, 1970; (8) = Ferber et al., 1972; (9) = Emmelot and Bos, 1969; (10) = Ladoulis et al., 1974; (11) = Smith et al., 1975

homogenization in hypotonic medium) 2) short duration of gradient ultracentrifugation 3) small contamination of plasma membranes with SER 4) good protein recovery and 5) high sialic acid to protein ratio in the preparations.

The drawbacks of our method are the following: 1) the membranes contain more DNA than other plasma membrane preparations and 2) mitochondria cannot be separated in an intact state.

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Preparation of Polysaccharide Derivatives Containing Amino Groups for Application in Affinity Chromatography

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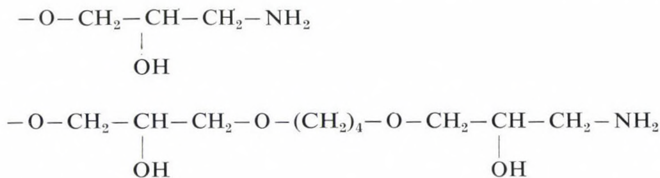
We describe here simple methods to introduce spacer arms with amino functional group into polysaccharides.



The technique includes the following steps: Compounds comprising epoxide group(s) are introduced into polymer supports to form the spacers. Then, reactive groups on the other side of the introduced spacers are reacted with ammonia. Since spacers are attached via ether linkage to the supports, the escape of ligands from specific sorbents can be prevented. Moreover, no charged groups arise on the carrier during the activation procedure, thus decreasing the nonspecific protein retention.

Introduction

To this time, the cyanogen bromide (CNBr) technique (Axén et al., 1967; Porath et al., 1967; March et al., 1974; March et al., 1974) has been the most widely used procedure to attach various ligands to polysaccharides. A serious disadvantage of this method is that anion exchanger groups appear on the support (Svenson, 1973; Wilchek et al., 1975). The affinity matrix having hydrophobic spacer arms and charged groups functions as a detergent. This results in the adsorption of a number of partly denatured proteins (Jost et al., 1974; Nishikawa, Bailon, 1975) leading to enhanced nonspecific protein retention which lessens the selectivity of isolation. In addition, the immobilized ligand leaks from the matrix due to the lability of the isourea bond formed during the introduction of the spacer (Parikh et al., 1974). This is an especially serious limitation for the use of affinity chromatography in isolation of hormone receptors or other proteins of very small (nanomolar) concentrations (Turkova, 1978).

The adsorbents prepared by the epoxide technique (Sundberg, Porath, 1974; Axén et al., 1975) exhibit much higher stability and lower nonspecific adsorption (Murphy et al., 1977) than the adsorbents mentioned above. However, preparation of spacer arms with amino functional groups by the epoxide technique has not been described yet. We have now developed a simple method to convert reactive groups, introduced into polysaccharide supports by the initial epoxidation step, into amino groups. Simultaneously, we have modified the condition of epoxidation described (Sundberg, Porath, 1974; Axén et al., 1975) to avoid formation of extended crosslinks on the carrier.

Materials and methods

Agarose (Sepharose 2B, 4B, 6B), AH-Sepharose 4B and Dextran T 500 were purchased from Pharmacia (Uppsala, Sweden), 1,4-bis (2,3-epoxy-propoxy) butane from EGA Chemie (Steinheim, GFR), cellulose (CF 11) from Whatman Co. (U.K.). All other chemicals were obtained from Reanal (Budapest, Hungary).

Amino content was determined by the method of Failla and Santi (1973).

To estimate the extent of crosslinks, aliquots of different matrices were hydrolyzed in 50% acetic acid at 75 °C and the solubilization times were measured. Unsubstituted Sepharose 4B was used as control material.

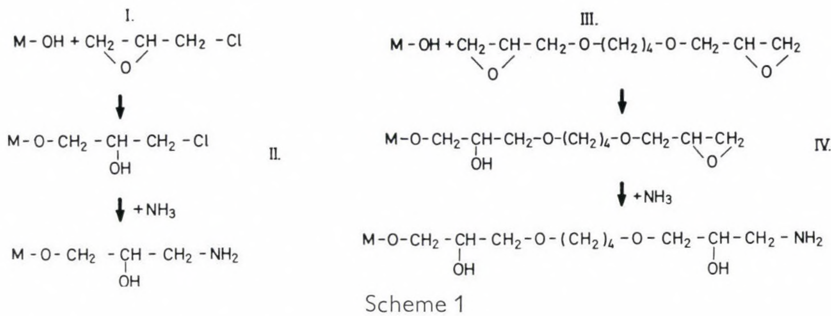
To determine the dry matter content, 5 ml gel was dried at 80 °C to constant weight.

Results and discussion

We have modified the reaction conditions of the initial epoxidation step described by Sundberg and Porath (1974) and Axén et al., (1975). We have considerably reduced the concentration of the epoxide compounds applied, the original amount of the reaction time, the alkalinity of the reaction media, and, in one case (Axén et al., 1975), the reaction temperature, too. By this means, the activation procedures became more economic, spacers could be introduced into the support in various amounts and more gentle reaction conditions were achieved than in the aforementioned procedures. This way we could avoid the formation of extended cross-links on the supports that may decrease their permeability for materials of high molecular weight. The polymer supports (designated as M – OH) were reacted with 1-chloro-2,3-epoxypropane (I), or 1,4-bis (2,3-epoxypropoxy)butane (III), and the products isolated (II, IV) were treated with ammonia to form amino groups (Scheme 1).

(i) Introduction of spacers into agarose using 1-chloro-2,3-epoxypropane:

To 5 g suction-dried Sepharose 4B suspended in 6.5 ml 0.25 M NaOH 0.33 ml 1-chloro-2,3-epoxypropane was added and the mixture was shaken at room temperature for 2 hrs. The modified agarose was washed with distilled water to neut-



Scheme 1

rality and traces of epoxide compounds were removed by washing with 15 ml acetone. After removing the acetone by suction, the gel was washed with water and allowed to stand in 7.5 ml 12 M ammonia solution overnight. This was followed by washing with water to neutrality and amino content was determined.

(ii) Introduction of spacers into agarose using 1,4-bis-(2,3-Epoxypropoxy) butane.

A sample of 5 g suction dried Sepharose 4B agarose was mixed with 6.5 ml 0.5 M NaOH solution and 1.25 ml 1,4-bis(epoxypropoxy) butane. The suspension was shaken at room temperature for 2 h. The modified gel was processed as described above. Both of these methods were carried out also on a 20-fold larger scale. By varying the amount of epoxide compounds during activation the final amount of amino groups can be regulated (Table 1). The introduction of spacers in higher amounts than reported here (315 μmol NH_2/g dried gel corresponding to about 11 μmol NH_2/ml settled gel) in unnecessary for affinity chromatography purposes.

We verified that the structure of the modified gels altered only slightly under the reaction conditions used. We hydrolyzed the matrices modified by various methods (Axén et al., 1967; Porath et al., 1967; March et al., 1974; Sundberg, Porath, 1974; Axén et al., 1975) in 50% acetic acid and measured the solubilization times (Table 2). The modified gels described in this paper dissolved during the

Table 1

Modification of agarose with various amounts of epoxide compounds

Epoxide compound	Reagent, 5 g wet gel	Amino content, μmol NH^2/g dried product
1-chloro-2,3-epoxypropane	0.33	315
	0.11	101
	0.055	54
1,4-bis(2,3-epoxypropoxy)butane	1.25	112
	0.42	41
	0.21	18

Table 2

Hydrolysis of the various modified agarose gels in acetic acid

	Control samples (Sepharose 4B)	Prepared by the CNBr technique (AH-Sepharose 4B)	Prepared by modification with 1-chloro-2,3-epoxy-propane		Prepared by modif-ication with 1,4-bis-(2,3-epoxypropoxy)-butane		
Solubilization time (hours)	1	>10	1[a]	>10[b]	1[a]	>10[b]	>10[c]

Samples of 0.3 ml of various gels were hydrolyzed at 75 °C in 5 ml 50% acetic acid and the solubilization times were measured

[a] Prepared as described in the text

[b] Prepared as described by Axén et al. (1975)

[c] Prepared by the procedure of Sundberg, Porath (1974)

same time as the control materials, indicating that extended crosslinks did not form on the carrier, whereas the products of the other procedures remained practically unchanged even after 10 h.

Our procedure is suitable for the introduction of amino groups into other polysaccharides such as dextran and cellulose provided that the dry matter weights are properly taken into account. When cellulose is used as material to be modified the procedure is the same as described above. Since dextran is water-soluble the recovery of the modified material was performed by dropping the reaction mixture into methanol of five-fold volume. The precipitate was filtered on sintered glass (type G-3), washed with methanol and acetone, then dried and precipitation was repeated. The product was recovered after each reaction step by the same procedure.

By applying the above methods hydrophilic spacer arms with amino functional groups can be connected to polysaccharide carriers through stable ether linkage. The amino derivatized polysaccharides can easily be converted into other derivatives containing different functional groups (Cuatrecasas, 1970) for immobilization of various ligands.

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Steroid Spectrum in Human Urine as Revealed by Gas Chromatography

IV. Changes in the Excretion of 16-Oxygenated Neutral Steroids by Children with 21-Hydroxylase Deficiency during Different Stages of Development

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Two 16α-hydroxy-C_{21}-steroids, three 16-oxygenated-C_{19} steroids, pregnened io and seven C_{19}-steroids were separated and quantitatively determined in the urine of 7 salt-loosing patients with congenital adrenal hyperplasia (deficient in C_{21}-hydroxylase), aged from 4 weeks to 13 years. Out of the twelve urine samples studied, 3 were collected before the beginning of corticoid treatment and 9 were obtained during corticoid therapy with a dose partially suppressing endogenous ACTH secretion.

Depending upon the extent of the suppression of endogenous ACTH secretion, the intensity of steroidogenesis showed great variation, and therefore, instead of evaluation on the basis of absolute amounts, steroids were divided into groups and characterized by their excretion ratio relative to 17α-hydroxy-pregnanolone. The excretion of 16-oxygenated-C_{21}- and -C_{19}-steroids dramatically decreased after the age of 8 weeks, which may be explained by the regression of the foetal zone of the adrenal gland and the decrease of the 16-hydroxylase activity of the liver. At the age of 3–6 years, the excretion of total C_{19}-steroids increased moderately and continuously, parallel to which a well-defined increase in the excretion of 16α-hydroxy-C_{21}-steroids was observed. In puberty the excretion of total C_{19}-steroids increased further; however, this was no more accompanied by an increase in C_{21}-16α-hydroxylation – on the contrary, a decrease of the latter was observed. The excretion of 16-oxygenated-C_{19}-steroids and that of the pregnenediol increased parallel with that of total C_{19}-steroids in puberty; this increase may be connected with the adrenarche.

It was established that in children with 21-hydroxylase deficiency, submitted to substitutive corticoid therapy, the changes in steroid metabolism characteristic both of the first months of infancy and of puberty essentially occur at times similar to the physiological ones. A difference was observed, however, between the ages of 3 and 6, inasmuch as the excretion of 16α-hydroxy-C_{21}-steroids increased. This pointed to a correlation between C_{21}-16α-hydroxylation and $C_{17,20}$-lyase in 21-hydroxylase deficiency.

Abbreviations: ACTH, adrenocorticotropic hormone; CHDMS, cyclohexanedimethanol succinate, stationary phase; DHA, dehydroepiandrosterone; DHA-S, dehydroepiand trosterone-sulphate; FSH, follicle-stimulating hormone; GC, gas chromatography; GC-MS, gas chromatography combined with mass spectrometry; IST, internal standard; LH, luteinizing hormone; Sp 2100, methyl-substituted polysiloxan, stationary phase; Sp 2250, methylphenyl-(50–50%)-substituted polysiloxan, stationary phase; RRT_{ch}, retention time relative to 5 α-cholestane; UR, unresolved components.

Introduction

In the course of puberty not only steroid production by the gonads is increased (gonadarche), but also the formation of adrenal androgens. The connection between the latter and the appearence of pubic hair was first recognized by Albright et al. (1942), who termed the maturation process of the adrenal gland as "adrenarche". The mechanism of adrenarche is not yet understood; it is not accounted for by an increased trop-hormone stimulation, as – unlike in the case of gonadotropins – the secretion rate of ACTH is not increased. This is indicated by a constant cortisol secretion relative to body weight during puberty (Migeon et al., 1963).

The maturation of the adrenals starts prior to the activation of the hypophysis-gonad axis, since the increase in the serum concentrations of DHA and DHA-S precedes the enhancement of FSH and LH secretion and also the first clinical signs of puberty by 2–3 years (Sizonenko, Paunier, 1975; Korth-Schutz et al., 1976). These observations also raised the possibility of a specific regulation of adrenal androgens. The synergistic effect of a great variety of hormones and of ACTH has been studied; however, the data on the correlation of hormone levels are contradictory and the synergistic effect is doubtful (Lee et al., 1975; Parker, Odell, 1979).

In untreated patients with 21-hydroxylase deficiency, chronic hypersecretion of ACTH necessarily leads to the hyperplasia of the adrenal gland, to hyperandrogenism and an early adrenarche. By means of corticoid treatment ACTH secretion may be decreased to the desired extent and the early adrenarche prevented.

The adrenal gland of the newborn exhibits hyperplasia as compared to infants and children because of the foetal zone. The foetal zone secretes DHA-S which is the precursor of oestrogens in the intrauterine foeto-placental unit (Diczfalusy, 1964). In the first weeks of infancy the adrenal gland keeps intensively secreting DHA-S, still this is not followed by an early adrenarche since the steroid-sulfokinase activity of the tissues and the 16 α-hydroxylase activity of the liver are enhanced and DHA-S is thus transformed into 16 α-hydroxy-DHA-S.

In our present work 16-oxygenated steroids were studied in enzymopathic children and infants of various ages, in whom androgen secretion was suppressed by corticoid treatment and early adrenarche prevented. Our observations indicate that between the age of 3 and 6 the moderate increase in androgen secretion by the adrenals is accompanied by an increase in the excretion of 16-hydroxy-C_{21}-steroids. This points to a possible functional relationship between 17–20-lyase and C_{21}-16α-hydroxylation.

Materials and methods

Reference steroids (Table 1.): Reference steroids were kindly supplied by Prof. D. N. Kirk (Medical Research Council, Steroid Reference Collection, London).

Table 1

Systematic names of the steroids studied

Systematic name	Trivial name	Number on the chromato-grams
3α-hydroxy-5α-androstan-17-one	androsterone	1
3α-hydroxy-5β-androstan-17-one	etiocholanolone	1
3β-hydroxy-5-androsten-17-one	dehydroepiandrosterone	3
3α-hydroxy-5α-androstane-11,17-dione	11-keto-androsterone	4
3α-hydroxy-5β-androstane-11,17-dione	11-keto-etiocholanolone	4
3α,11β-dihydroxy-5α-androstan-17-one	11-hydroxy-androsterone	7
3α,11β-dihydroxy-5β-androstan-17-one	11-hydroxy-etiocholanolone	6
5-androstene-3β,17β-diol	androstenediol	—
3β,16α-dihydroxy-5-androsten-17-one	16α-hydroxy-DHA	11
3β,16β-dihydroxy-5-androsten-17-one	16β-hydroxy-DHA	11
3β,17β-dihydroxy-5-androsten-16-one	16-keto-androstenediol	12
5β-pregnane-3α,20α-diol	pregnanediol	8
5-pregnene-3β,20α-diol	pregnanediol	10
3α,17α-dihydroxy-5β-pregnan-20-one	17α-hydroxy-pregnanolone	9
5β-pregnane-3α,17α,20α-triol	pregnanetriol	13
3α,17α,20α-trihydroxy-5β-pregnan-11-one	11-keto-pregnanetriol	16
3β,16α-dihydroxy-5-pregnen-20-one	16α-hydroxy-pregnenolone	—
3β-hydroxy-5,16-pregnadien-20-one	16-dehydro-pregnenolone	5
5-pregnene-3β,17α,20α-triol	pregnenetriol	14
5-pregnene-3β,16α,20α-triol	16α-hydroxy-20α-dihydro-pregnenolone	17
5-cholesten-3β-ol	cholesterin	15

Material studied: Urine collected during 24 hours from 7 salt-loosing infants and children with 21-hydroxylase deficiency was used for the experiments. The clinical data of the patients are summarized in Table 2.

For patients O. I., M. V. and K. Z., enzyme deficiency was verified by gas chromatographic determinations of the steroid spectrum; for the other children the diagnosis was confirmed and the substitution therapy checked.

Analytical procedures: The extraction of steroids, the formation of derivatives and gas chromatographic analyses were carried out according to a modified version of our method described earlier (Kecskés, Juricskay, 1975), in the following way:

a) *Processing of the urine by acid hydrolysis and toluene extraction:* One fifth of the daily urine was hydrolyzed under acidic conditions and simultaneously extracted by toluene in two separate steps. First the pH of the urine sample was adjusted to 1 by adding 60% (v/v) sulfuric acid, then 0.5 volume of toluene was layered on top and the sample was refluxed by boiling for 20 min. After the separation of the organic phase, 0.1 volume of 60% sulfuric acid was added to the urine and the mixture was again refluxed for 20 min. After separation, the two organic

Table 2

Clinical history of children with 21-hydroxylase deficiency

Case	Age, year (week)	Sex	Treatment mg/day before GC. anal.	Pubertal stage *a*	Salt-loosing syndrome	Clinical observation
O. I.	(4)	M	none	P 1	++	macrogenitosomia
O. I.	(6)	M	12.5 mg[b]	P 1	++	macrogenitosomia
M. V.	(8)	F	none	P 1	++	pseudo-hermaphroditism type II[d]
K. Z.	(10)	M	none	P 1	+++	none
K. T.	1 1/2	M	5 mg[c]	P 1	+	none
K. T.	4	M	5 mg[c]	P 1	+	none
I. T.	2 1/2	M	5 mg[c]	P 1	++	macrogenitosomia
I. T.	3 1/2	M	2.5 mg[c]	P 1	++	macrogenitosomia
V. Z.	6	M	5 mg[c]	P 2	±	none
T. K.	11	F	20 mg[c]	P 3	±	pseudo-hermaphroditism type III[d]
T. K.	12	F	20 mg[c]	P 3	±	pseudo-hermaphroditism type III[d]
T. K.	13	F	10 mg[c]	P 3	±	pseudo-hermaphroditism type III[d]

[a] On the basis of pubic hair according to *Tanner* (1962)
[b] adresone (*Organon*)
[c] prednisolon (*Richter*)
[d] genital configuration according to *Prader* (1958)

phases were combined, washed with 2 N NaOH and with distilled water, dried over sodium sulfate and the solvent evaporated.

Acetylation: the dry extract was dissolved in 0.2 ml of pyridine, and 0.1 ml was acetylated for 12 hours at room temperature by 0.5 ml acetic anhydride, and the sample was then evaporated under CO_2 in a 50 °C water bath. The acetylated extract was dissolved in a mixture of ethanol : benzene (1 : 1) and used for gas chromatographic analysis.

b) *Processing of the urine by enzymatic hydrolysis and ether extraction:* One-fifth of the daily urine was hydrolyzed for 24 hours at 37 °C in 0.1 volume 2 M acetate buffer (pH 5.2) by 6 mU-glucuronidase, 2 mU arylsulphatase (*Helix pomatia*; Calbiochem-Behring Co.) and 0.5 mg streptomycin per ml reaction mixture. The hydrolysate was extracted by 2×2 volumes of ether free of peroxide, the combined extracts were washed with 2 N NaOH, 1 N acetic acid and distilled water and evaporated. Further processing was carried out as described in a).

c) *Gas chromatography*

I. Instrument: Packard 7300 dual column gas chromatograph.

II. Columns: Glass columns of a length of 1.82 m (inner diameter 2 mm), filling:

Column "A" with combined filling: at the inlet end, 78 cm 3% Sp 2250 stationary phase on Supelcoport 100/120 mesh support; at the detector end, 102 cm 3% Sp 2100 stationary phase on Supelcoport 80/100 mesh support.

Column "B": 3% Sp 2100 stationary phase on Supelcoport 80/100 mesh support.

Column "C": 2% CHDMS stationary phase on Gas Chrom Q 80/100 mesh support.

Detector: Dual flame ionization detector (its temperature was identical with the final temperature of the column).

Carrier gas: N_2 of high purity, flow rate 40 ml/min.

Conditions of analysis: On-column injection technique, heated inlet (its temperature was identical with the final temperature of the column). Simultaneous analysis on columns "A" and "B", isotherm stretch at 250 °C (its duration was three times the retention time of 5α-cholestane injected previously), followed by increasing the temperature to 270 °C at a rate of 1 °C/min. On column "C", isotherm analysis at 230 °C.

Table 3

Identification of peaks on chromatograms

Peak No.	Trivial name of steroids	*"A" column* Sp 2250/Sp 2100 RRT ch	*"B" column* Sp 2100 RRT ch	*"C" column* CHDMS RRT ch
1	androsterone	0.82[c]	0.53[c]	2.58
2	etiocholanolone	0.82[c]	0.54[c]	2.90
3	DHA	0.96	0.58	3.28
4	11-keto-etiocholanolone + 11-keto-androsterone	1.14–1.20[d]	0.64[c]	
5	16-dehydro-pregnenolone[a]	1.32	0.88[d]	
6	11-hydroxy-etiocholanolone	1.48	0.84[d]	
7	11-hydroxy-androsterone	1.56	0.84[d]	
8	pregnanediol	1.74[d]	1.28[d]	
9	17α-hydroxy-pregnanolone	1.76[d]	1.18	
10	pregnenediol	2.02	1.34[d]	
11	16α/β-hydroxy-DHA	2.36	1.22–1.28[d]	
12	16-keto-androstenediol	2.46	1.24[d]	
13	pregnanetriol[b]	2.94	1.85	
14	pregnenetriol[b]	3.13[d]	1.95	
15	cholesterin	3.26[d]	2.66[d]	
16	11-keto-pregnanetriol[b]	3.96	2.34	
17	16α-hydroxy-20α-dihydro-pregnenolone	4.12	2.62[d]	

[a] decomposed from 16α-hydroxy-pregnenolone
[b] estimated after enzyme hydrolyzis
[c] common peak
[d] unresolved peak

III. Identification: In the case of children with 21-hydroxylase deficiency, the steroid spectrum cannot be resolved on any column to the desired extent; for this reason three different columns had to be used. The steroids separated in the form of acetyl derivatives, identified (and also quantitatively determined) on the basis of their retention time relative to 5α-cholestane, are shown in Table 3. When necessary, prefractionation by paper chromatography in the Bush B_5 system was also carried out in order to check specificity.

IV. Quantitative determination: To the acetylated urine extract 30 – 150 μg of cholesteryl-amylether was added prior to analysis. The area under the peaks was calculated by multiplying the height by the width measured at half of the height. The response factor of the individual steroids was determined with the help of known amounts of reference steroids. Of the reference steroids only steroids $C_{19}O_2$ and $C_{21}O_2$ showed an area to mass ratio identical with that of cholesteryl-amylether if calculated for free steroids. The area/mass ratio of $C_{19}O_3$ and $C_{21}O_{3-4}$ steroids was lower than that of an identical quantity of cholesteryl-amylether: it amounted to 86 – 21 % of the latter. When quantitating urine extracts, the reciprocal of the response factor of these steroids relative to cholesteryl-amylether was used as correction factor (Table 4): the area of the steroid in question was divided by that of cholesteryl-amylether and this ratio was multiplied by the correction factor. From this value steroid excretion in μg/24 hours was calculated, taking into consideration the amount of cholesteryl-amylether added to the extract and the extent of dilution.

Table 4

Reciprocal response factors of reference steroids to cholesteryl-amylether

Peak No.	Trivial name of steroids	"*A*" *column* Sp 2250/Sp 2100	"*B*" *column* Sp 2100
1	androsterone	1.0	—
2	etiocholanolone	1.0	—
3	DHA	1.0	—
4	11-keto-etiocholanolone + 11-keto-androsterone	—	1.16
5	16-dehydro-pregnenolone	4.69	—
6	11-hydroxy-etiocholanolone	1.18	—
7	11-hydroxy-androsterone	1.18	—
8	pregnanediol	1.0	1.00
9	17α-hydroxy-pregnanolone	—	1.85
10	pregnenediol	1.0	—
11	16α/β-hydroxy-DHA	1.46	1.46
12	16-keto-androstenediol	1.14	1.14
13	pregnanetriol	2.70	—
14	pregnenetriol	2.80	—
15	11-keto-pregnanetriol	2.70	—
16	16α-hydroxy-20α-dihydro-pregnenolone	4.00	—

The lowest traceable amount of material with a response factor of 1.0 was 1 μg of steroid/sample or 10 μg/day. In case of a response factor lower than 1.0, the above values should be multiplied by the reciprocal of the response factor.

d) *Heat and acid stability of 16-oxygenated steroids at pH 1.0:* From 0.1% stock solutions in ethanol, 100 μg steroid was added to 30 ml was distilled water, the pH was adjusted to 1.0 and the sample was refluxed for 20 min without layering toluene on top; in this way the steroid studied was exposed to maximum effect of heat and acid. Toluene was then layered on the sample and it was extracted by refluxing for another 20 min. The toluene extract was processed and analyzed on column "A". The area ratios, relative to equal amounts of cholesteryl-amylether, are listed in Table 5, compared to reference steroids which had been only acetylated. From these data the minimal recoveries of 16-hydroxylated steroids were calculated, which are also shown in Table 5.

Table 5

Acid and heat stability of 16-oxygenated steroids at pH 1

Trivial name of steroids	Amount μg	Q area steroid/area IST		Recovery, %
		only GC	GC after 20 min boiling at pH 1	
16α-hydroxy-DHA	100	0.71	0.10	14
16β-hydroxy-DHA	100	0.71	0.26	37
16-keto-androstenediol	100	0.88	0.09	10
16α-hydroxy-pregnenolone	100	0.22	0.19	86
16α-hydroxy-20α-dihydro-pregnenolone	100	0.25	0.14	56

Results

Figure 1 shows the steroid spectrum of an infant with 21-hydroxylase deficiency in the first weeks of life. The urine extract of O.I., a 4-week-old infant gave the upper chromatogram on column "A" (Sp 2250/Sp 2100) and the lower chromatogram on column "B" (Sp 2100). The dominant peak in the upper chromatogram is 16-dehydropregnenolone (5), which is derived from 16α-hydroxypregnenolone by dehydration. The relative retention time of the same steroid on the lower chromatogram is 0.88; however, it cannot be resolved from the neighbouring peaks, therefore its identification number is not shown, only its position is marked "UR". On the upper chromatogram, the peaks of the still identifiable 16-oxygenated steroids, i.e. 16α/β-hydroxy-DHA (11) and 16α-hydroxy-20α-dihydropregnenolone (17) are considerably smaller. The following components could be quantitatively determined: androsterone + etiocholanolone (1 and 2), 11-hydroxyetiocholano-

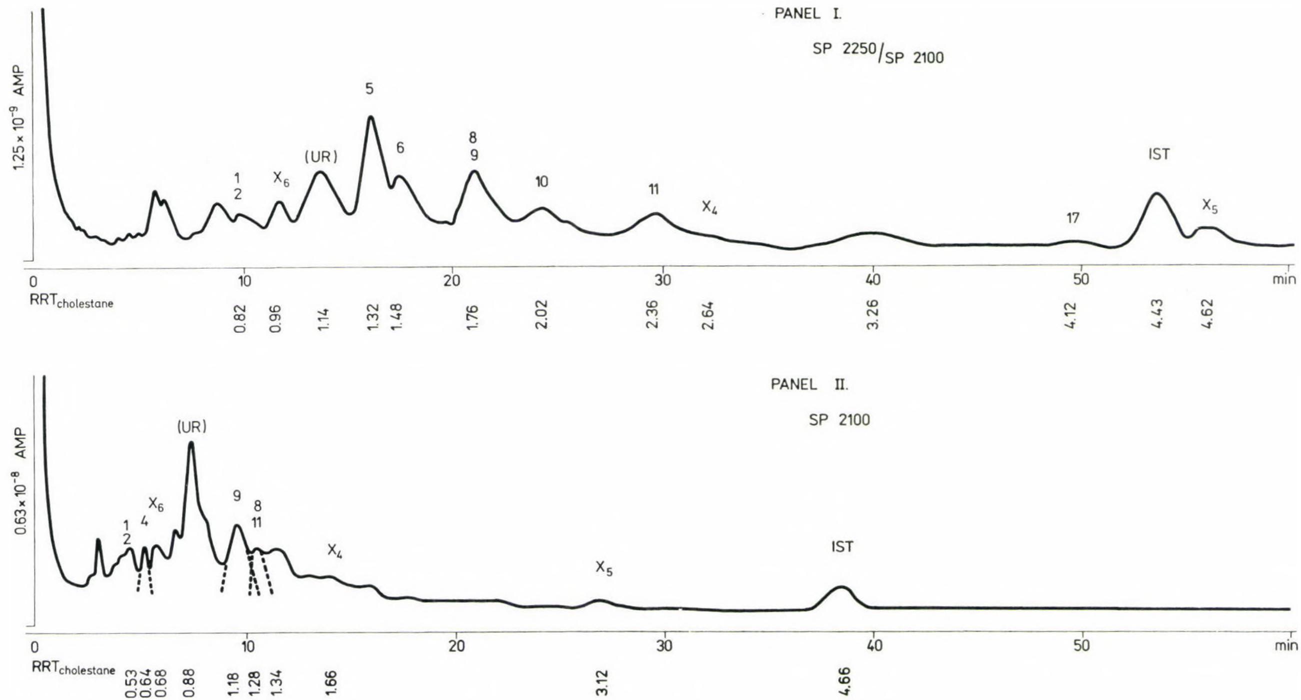

Fig. 1. Simoultaneous gas chromatographic analysis of urinary steroids as acetyl derivatives obtained from a 4-week-old infant with 21-hydroxylase deficiency. Conditions: acid hydrolysis. Panel I, Sp 2250/Sp 2100 column; Panel II, Sp 2100 column. The compounds are identified in Table 3. x_4, x_5, x_6 are unidentified peaks, UR are unresolved components

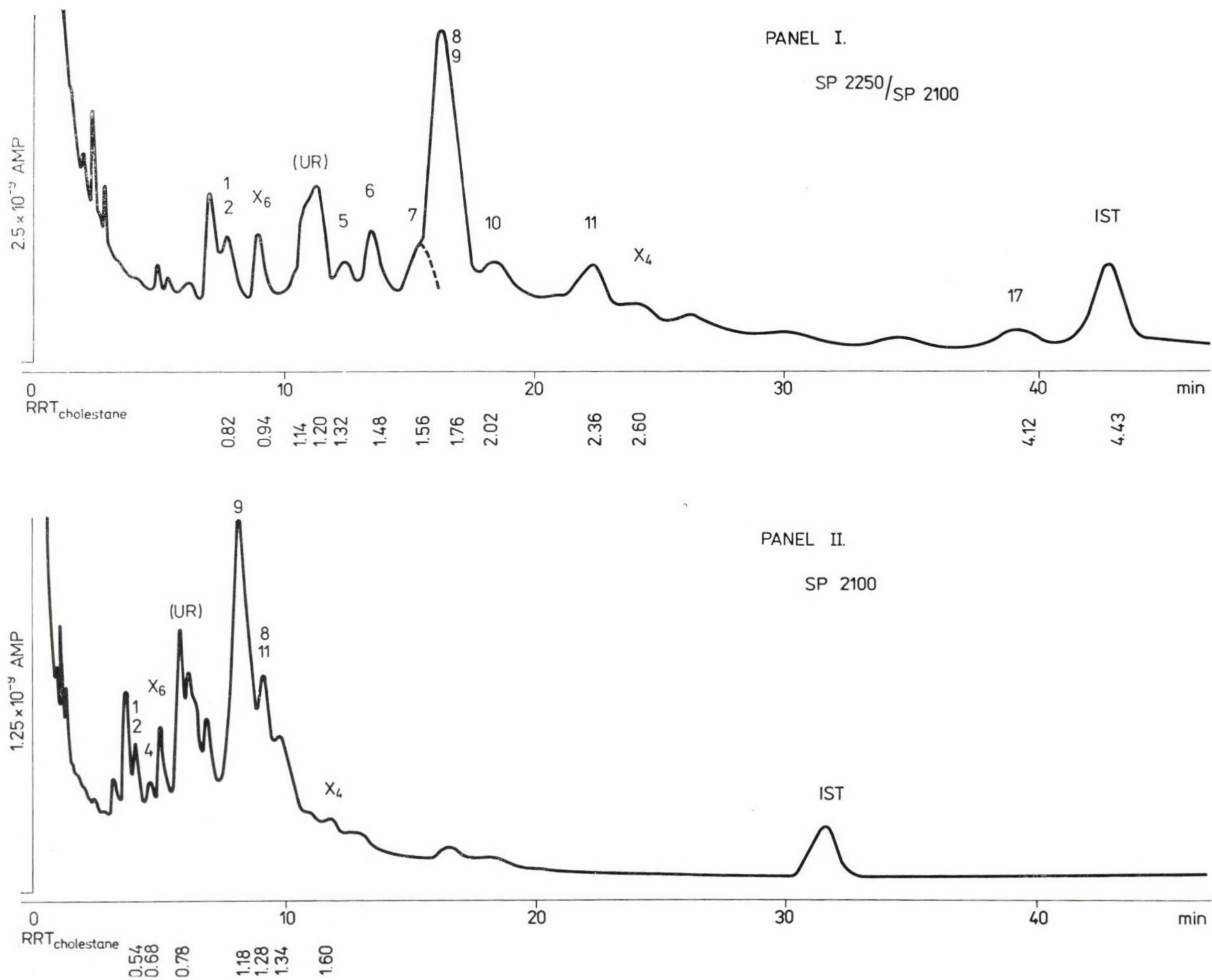

Fig. 2. Simultaneous gas chromatographic analysis of urinary steroids as acetyl derivatives obtained from a 2 1/2-year-old child with 21-hydroxylase deficiency. Conditions: acid hydrolysis. Panel I, Sp 2250/Sp 2100 column; Panel II, Sp 2100 column. The compounds are identified in Table 3. x_4, x_6 are unidentified peaks, UR are unresolved components

lone (6), pregnenediol (10). The material in the peak with a relative retention time of 1.13 could not be quantitated, since in addition to 11-keto-androsterone it also contained androstenediol and various pregnanolone isomers. Similarly the peak with a relative retention time of 1.74 (8 and 9) contained a mixture of 17α-hydroxy-pregnanolone and pregnanediol and therefore could not be quantitated. The component with a relative retention time of 0.96 is virtually identical with DHA. However, the peak corresponding to DHA is absent in the lower chromatogram. It could not be traced on a CHDMS column either, and therefore we do not consider it as DHA.

In the lower chromatogram, 11-keto-androsterone together with 11-keto-etiocholanolone (4), furthermore 17α-hydroxypregnanolone (9) were identified and quantitated. Pregnanediol (8) is the only one from the steroids studied that could not be separated on either column used. Therefore its amount was determined indirectly and considered only semiquantitative. The following calculation was done: from the area of the common peak of pregnanediol and 16α/β-hydroxy-DHA, measured on column "B" (8 and 11), the area corresponding to that occupied by 16α/β-hydroxy-DHA on column "A" was substracted and the remaining area gave the amount of pregnanediol. On both chromatograms, 3 non-identified peaks are shown, marked x_4, x_5 and x_6. The identification of these peaks by means of GC-MS is in progress.

The steroid spectrum of I. T., a 2 1/2-year-old child, is presented in Fig. 2. This type of chromatogram is characteristic of the age between half a year and 6 years. On the upper chromatogram the dominant peak contains 17α-hydroxy-pregnanolone together with pregnanediol (8 and 9), therefore 17α-hydroxy-pregnanolone (9) was evaluated from the lower chromatogram. From 16-oxygenated steroids 16-dehydro-pregnenolone (5) and 16α/β-hydroxy-DHA (11) were identified. In addition, the following components could be quantitated: androsterone + etiocholanolone (1 and 2), 11-hydroxy-etiocholanolone (6), 11-hydroxy-androsterone (7), pregnenediol (10).

From the lower chromatogram, 11-keto-androsterone + 11-keto-etiocholanolone (4), 17α-hydroxy-pregnanolone (9) and pregnanediol (8) were identified for quantitative determination.

Figure 3 shows the chromatogram of the urine extract of I. T., after enzymatic hydrolysis. For this experiment the same urine sample was used as above. On this chromatogram pregnanetriol (13), 11-keto-pregnanetriol (16) and 5-pregnenetriol (14) were also identified. Owing to their high sensitivity to acids and heat, these steroids cannot be studied after acid hydrolysis (Fig. 2). The non-identifiable peaks (x_4, x_6) are also present in this chromatogram, which means that they are not to be considered as artefacts of acid hydrolysis.

Our quantitative results are listed in Table 6. In order to illustrate the order of magnitude of the steroid values, those of two healthy newborns and of 6 healthy children of ages similar to those of the enzymopathic children are presented in Table 7. A comparison of the data in the two Tables leads to the following conclusions:

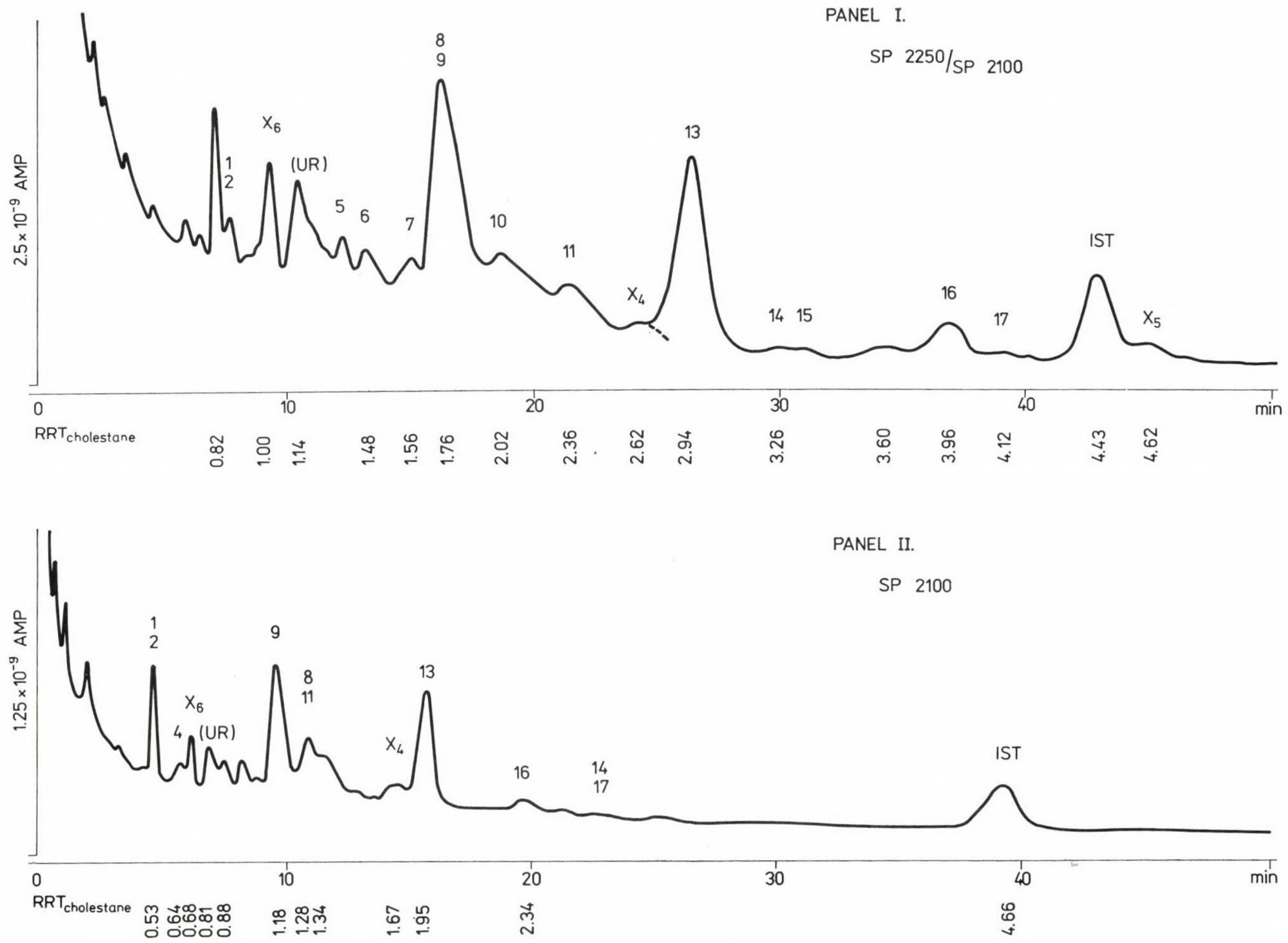

Fig. 3. Simultaneous gas chromatographic analysis of urinary steroids as acetyl derivatives obtained from a 2 1/2-year-old child with 21-hydroxylase deficiency. Conditions: enzyme hydrolysis. Panel I, Sp 2250/Sp 2100 column; Panel II, Sp 2100 column. The compounds are identified in Table 3. x_4, x_6 are unidentified peaks, UR are unresolved components

Table 6

The urinary excretion of steroids by infants and children

Name, age		O. I. 4 weeks	O. I. 6 weeks	M. V. 8 weeks	K. Z. 10 weeks
Steroid					
1	androsterone	225	150	20	57
2	etiocholanolone	—	—	169	17
3	DHA	—	—	—	—
4	11-keto-androsterone + 11-keto-etiocholanolone	2175	1172	290	110
5	16-dehydro-pregnenolone	9918	13460	1078	450
6	11-hydroxy-etiocholanolone	—	—	118	83
7	11-hydroxy-androsterone	—	—	153	—
8	pregnanediol	68	—	221	133
9	17α-hydroxy-pregnanolone	3688	6811	2499	1407
10	pregnenediol	780	1345	572	137
11	16α/β-hydroxy-DHA	1357	549	1267	161
12	16-keto-androstenediol	—	1030	—	128
13	pregnanetriol	2044	*a*	*a*	1243
14	pregnenetriol	—	*a*	*a*	42
15	11-keto-pregnanetriol	3080	*a*	*a*	941
16	16α-hydroxy-20α-dihydro-pregnanolone	660	125	223	—
I	Total of 16-oxygenated C_{21} steroids	10578	13585	1301	450
II	Total of 16-oxygenated C_{19} steroids	1357	1579	1267	289
III	Total of C_{19} steroids	3757	2901	2017	556
	Ratio I/9	2.87	1.99	0.52	0.32
	Ratio II/9	0.37	0.23	0.51	0.21
	Ratio III/9	1.02	0.43	0.81	0.40
	Ratio 10/9	0.21	0.20	0.23	0.10

a not determined

1. The enzymopathic children excreted a considerable amount of 17α-hydroxy-progesterone metabolite even under substitutive corticoid treatment, which means that at the time of our investigation the treatment was not effective enough. In this case an increased amount of corticoid should be administrated. In the case of healthy children, 17α-hydroxy-pregnanolone is traceable from the age of 6, and its amount is significantly lower than the values in the enzymopathic group.

2. In enzymopathic children, 16-dehydro-pregnenolone, representing 16α-hydroxy-pregnenolone was the most abundant metabolite before the age of 10

with C_{21}-hydroxylase deficiency ($\mu g/24\ h$)

K. T. 1 1/2 years	I. T. 2 1/2 years	I. T. 3 1/2 years	K. T. 4 years	V. Z. 6 years	T. K. 11 years	T. K. 12 years	T. K. 13 years
205	38	1044	11	405	226	640	86
435	222	—	82	945	216	500	204
—	—	—	—	540	180	170	100
603	250	412	432	1322	127	408	244
3189	1219	3798	1238	561	375	469	164
944	—	—	—	—	—	83	130
873	519	212	197	556	177	307	118
234	584	—	476	1323	218	508	138
14171	8325	9480	3780	8824	2472	6556	805
900	300	552	280	90	104	750	125
963	722	—	403	394	186	876	219
—	—	1146	—	—	—	—	—
8802	11826	*a*	3274	*a*	7668	4428	—
—	—	*a*	—	*a*	—	80	144
2160	2754	*a*	1612	*a*	3261	1404	—
2000	1080	1512	400	3120	—	560	—
5189	2299	5310	1638	3681	375	1029	164
963	722	1146	403	394	186	876	219
4023	1751	2814	1125	4162	1112	2984	1101
0.37	0.28	0.56	0.43	0.42	0.15	0.16	0.20
0.07	0.09	0.12	0.11	0.04	0.08	0.13	0.27
0.28	0.21	0.30	0.30	0.47	0.45	0.46	1.37
0.06	0.04	0.06	0.07	0.01	0.04	0.11	0.16

weeks. However, a small amount also was traceable later. In the healthy group this steroid occurred in significant amounts only in infants.

3. The urine of children with enzyme deficiency contains higher amounts of 16α/β-hydroxy-DHA and also 16α-hydroxy-20α-dihydro-pregnenolone. This latter could be traced in healthy children between the ages of 3 and 6.

4. The amount of total C_{19}-steroids was manifold higher in the enzymopathic children submitted to underdosed substitutive treatment than in the healthy group.

5. DHA is not detectable in either group before the age of 6 years. With one exception, androsterone and etiocholanolone were present in higher amounts

Table 7

The urinary excretion of steroids by healthy infants and children (μg/24 h)

Name, age		S. I. 6 days	Sz. G. 6 days	Sz. D. 3 years	K. J. 2 1/2 years	K. E. 6 years	G. J. 6 years	O. Cs. 11 years	H. K. 13 years
Steroid									
1	androsterone	28	—	—	10	80	28	783	860
2	etiocholanolone	—	—	—	40	40	32	576	997
3	DHA	—	—	—	—	—	18	174	89
4	11-keto-androsterone + 11-keto-etiocholanolone	88	—	28	39	136	25	123	260
5	16-dehydro-pregnenolone	966	180	35	—	—	—	—	—
6	11-hydroxy-etiocholanolone	—	—	—	—	—	—	—	—
7	11-hydroxy-androsterone	—	—	60	—	97	62	179	203
8	pregnanediol	—	—	35	11	—	—	108	442
9	17α-hydroxy-pregnanolone	—	—	—	—	93	67	—	117
10	pregnenediol	42	40	47	68	181	94	281	170
11	16α/β-hydroxy-DHA	169	—	—	—	—	—	—	—
12	16-keto-androstenediol	257	95	52	32	143	142	100	131
13	pregnanetriol	*a*	*a*	*a*	*a*	*a*	*a*	*a*	*a*
14	pregnenetriol	*a*	*a*	*a*	*a*	*a*	*a*	*a*	*a*
15	11-keto-pregnanetriol	*a*	*a*	*a*	*a*	*a*	*a*	*a*	*a*
16	16α-hydroxy-20α-dihydro-pregnenolone	—	—	42	25	64	—	—	—
I.	Total of 16-oxygenated C_{21} steroids	966	180	77	25	64	—	—	—
II.	Total of 16-oxygenated C_{19} steroids	426	95	52	32	143	142	100	131
III.	Total of C_{19} steroids	542	95	213	225	617	307	1935	2540

a not determined

in the children submitted to underdosed substitution treatment than in the healthy ones of similar age.

In our view, the absolute values of the steroid excretion of the children with 21-hydroxylase deficiency cannot be directly compared with each other, since these values primarily depend upon the effectivity of the substitutive corticoid treatment and as such reflect the extent of endogenous ACTH stimulation. In order to neutralize this factor, the most important steroids were combined in groups and their ratio relative to 17α-hydroxy-pregnanolone calculated. These

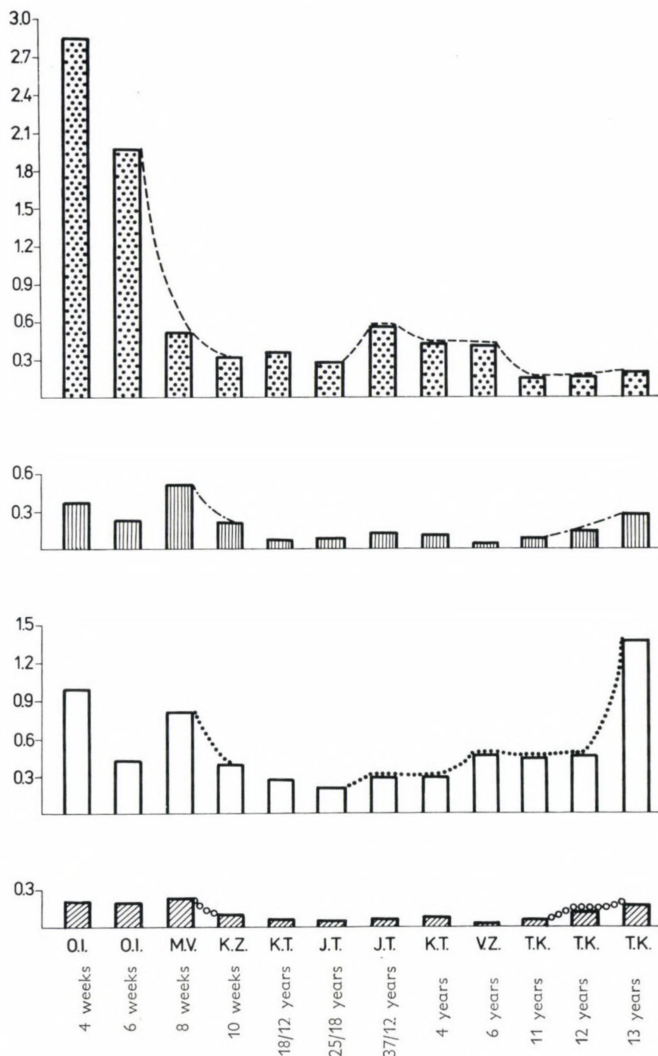

Fig. 4. The ratio of some steroid groups relative to 17α-hydroxy-pregnanolone excreted in the daily urine by children with 21-hydroxylase deficiency during different stages of development. — — — C_{21}-16-OH steroids/17-OH-Pg; — · — · — C_{19}-16-OH steroids/17-OH-Pg; · · · · □ total C_{19}-steroids/17-OH-Pg; ○ ○ ○ 5-Pd/17-OH-Pg

data are shown in Fig. 4. It is clear that after the 8th week 16-hydroxylating activity rapidly decreases both for C_{21}- and C_{19}-steroids (due to a decrease of the 16-hydroxylase activity of the liver), and the ratio of total C_{19}-steroids also decreases, indicating a decrease of the 17–20-lyase activity of the adrenals. The decrease of the pregnenediol ratio is indicative of the reduction of the pregnenolone pool of the or-

ganism. It seems to be significant that the 16-hydroxy-C_{21} ratio changes parallel with the total C_{19} ratio between the ages of 3 and 6. This points to a relationship between C_{21}-16α-hydroxylation and $C_{17,20}$-lyase activity. The same is not observed in the case of the ratio of 16-hydroxy-C_{19}-steroids, which begins to increase only in puberty parallel with the ratio of total C_{19}-steroids, similarly to the ratio of pregnenediol, which is lower between the ages of 1 and 11 years and increases only after the age of 12.

Discussion

Earlier we have found that the diagnostic value of 17α-hydroxy-pregnanolone determination by gas chromatography equals that of pregnanetriol in 21-hydroxylase deficiency of the virilizing type (Kecskés et al., 1975) and is also suitable for the verification of the enzyme deficiency in salt-loosing infants (Kecskés, 1978). However, the methyl-silicone stationary phase applied in the above studies proved to be inadequate for the separation of $\Delta 5-3\beta$,16-dihydroxy-steroids since resolution from other steroids was unsatisfactory (Table 3). Therefore we applied a combined column prepared from 3% Sp 2250 and 3% Sp 2100. From this column 16α-hydroxy-DHA and 16β-hydroxy-DHA are eluted in a common peak, while other 16-oxygenated steroids are obtained in a separate peak, free of other steroids. The combined column is stable, its ability to withstand heat and its life-span are identical with those of the methylsilicone (Sp 2100) column. The latter, however, is still necessary for the identification of 17-hydroxy-pregnanolone, 11-keto-androsterone and 11-keto-etiocholanolone, and is also advantageous because of simultaneous chromatography. A comparison of the two chromatograms gives great diagnostic certainty in the identification of the peaks and we only rarely had to recur to prefractionation by paper chromatography to ensure specificity. The CHDMS column was applied for the separation of androsterone and etiocholanolone (Juricskay, Kecskés, 1978).

The adrenals of foetuses and 1 – 2 month-old infants secrete high amounts of $\Delta 5-3\beta$-hydroxy-steroids and these are hydroxylated mainly by the enzymes of the liver (Solomon et al., 1967; Reynolds et al., 1969). However, both pregnenolone and DHA may be 16α-hydroxylated also in the adrenals (Villee et al., 1962). According to literature data the amount in the urine of $\Delta 5-3\beta$, 16-dihydroxy-steroids of infants significantly decreases after the first weeks and is no more detectable after the fifth month (Reynolds, 1965a). According to Reynolds (1965b), in enzymopathies of the 21-hydroxylase deficient type especially 16α-hydroxy-pregnenolone is excreted in high amounts, the quantity excreted being tenfold higher than that excreted by healthy infants. After the fifth month 16α-hydroxy-pregnenolone was present in the urine only in 15% of the cases studied by this author; upon the administration of ACTH, however, it appeared in all of the patients. Our results are similar to those of Reynolds (1965b) regarding the first months of infancy: it was after the 8th week that we observed a significant reduction in 16-hydroxylated steroids. The rapid decrease is caused by changes in the enzyme pattern of

the liver characterized by a dramatic decrease of 16-hydroxylating activity (Reynolds, 1966). However, we could detect small amounts of 16-hydroxy-steroids also after the fifth month. Others (Bongiovanni, 1966; Fortherby et al., 1957) isolated these steroids also at adult age, from high amounts of urine collected from several persons. We note that Reynolds (1965b) applied separation by paper chromatography followed by photometry.

According to our observations, the excretion ratio of both 16-hydroxy-C_{21}-steroids and total C_{19}-steroids relative to 17α-hydroxy-pregnanolone increase between the ages of 3 and 6 years, that is, the change in C_{21}-16α-hydroxylation and that in 17–20-lyase activity exhibit an identical tendency before prepuberty. The role of 16α-hydroxy-pregnenolone in steroidogenesis is unknown; this steroid may be considered as a metabolite rather than a hormone precursor. *In vivo* perfusion studies carried out on a foetus in the middle third of pregnancy were unsuccessful in demonstrating the transformation of 16α-hydroxy-pregnenolone into 16α, 17α-dihydroxy-pregnenolone or 16α-hydroxy-DHA (Reynolds et al., 1969).

16α-Hydroxy-progesterone inhibits testosterone production from 17α-hydroxy-progesterone in the testes by competitively inhibiting 17,20-lyase (Inano, Tamaoki, 1978). No similar studies have been carried out on adrenals. In the years preceding prepuberty the secretion of androgens by the adrenals is low in healthy children, i.e. the secretion of androgens and glucocorticoids is physiologically dissociated. The mechanism involved is unknown (Parker, Odell, 1979). The cause may be anatomical, i.e. insufficient development of the zona reticularis, but other functional regulatory factors may also be envisaged. Such a factor may be indicated by a recent observation (Kecskés et al., 1980): in healthy children pregnenediol excretion is low between the ages of 4–6 years and is increased in prepuberty, in a way similar to the increase in the serum concentration of DHA and DHA-S. The role of the decrease of C_{21}-16α-hydroxylase activity may also be considered to be responsible for the increase in pregnenediol excretion after the 6th year. However, this possibility is only speculative so far as the conditions of C_{21}-16-hydroxylation in healthy children are unknown. In our patients with 21-hydroxylase deficiency, the increase in pregnenediol excretion was observed only from the age of 11, simultaneously with an increase in the ratio of total C_{19}-steroids, i.e. at the onset of adrenarche.

The excretion of C_{19}-16-oxygenated steroids is also increased in puberty. 16α-hydroxy-DHA is the most significant precursor of oestriol in pregnancy, its role, however, in states other than pregnancy is unknown. Unlike DHA it has no androgenic effect (Prunty, 1966). 16β-hydroxy-DHA is also formed in high amounts in the foetus. Under physiological conditions it is found in the urine of adults in quantities considerably lower than the 16α-isomer. The possibility of its pathogenic role in one form of hypertonia has been raised (Lidlle, Sennett, 1975). Recent investigations, however, have not supported the salt-retaining effect of this steroid (Gomez-Sanchez et al., 1976; Higgins et al., 1977). Thus its suggested pathogenic role remains speculative. Our patients were all salt-loosing; in 21-hydroxylase deficiency salt loss is accounted for by a combined effect based on an overproduc-

tion of aldosterone antagonists (progesterone, 17-hydroxy-progesterone) and a decrease of the aldosterone synthetizing ability. At first the sodium-uretic effect of 16α-hydroxy-pregnanolone was suggested (Neher et al., 1959). However, later this assumption could not be verified (George et al., 1965). Still the sodium-uretic effect of 16α-hydroxy-progesterone in itself seems probable: Janoski et al. (1969) describe that in two salt-loosing children (aged 11 and 15 years), stoppage of suppressive glucocorticoid treatment resulted in the appearance of symptoms of salt-loss and a 3- and 30-fold increase, respectively, in the secretion rate of 16α-hydroxy-progesterone, while the secretion rate of 16α-pregnenolone remained constant.

The methylketon side chain of 16α-hydroxy-pregnanolone inhibits the acetylation of the 16α-hydroxy group. Due to the free hydroxyl group, at the temperature of gas chromatography the molecule is dehydrated and thereby transformed into 16-dehydro-pregnanolone. Another possibility for transformation is the formation of isopregnanolone by intestinal bacteria (Calvin, Lieberman, 1962). By our present separation techniques neither of these products can be separated from the pregnanolone complex. Therefore we have no data on the secretion of 16α-hydroxy-pregnanolone in these experiments.

To sum up, the multicomponent analysis of steroids on combined Sp 2250/Sp 2100 column of the acetylated urine extracts of salt-loosing infants and children with 21-hydroxylase deficiency yields information not only about the metabolites of 17α-hydroxy-progesterone which are of diagnostic value but also about the 16-oxygenated (3β-OH,Δ5) steroids dominant in the steroid metabolism of infants aged 1–2 month. The dramatic decrease of the amount of the latter occurs also in infants with 21-hydroxylase deficiency treated with corticosteroids at the age of 1–2 months, indicating a regression of the foetal zone of the adrenals and a change in the enzyme pattern of the liver. However, these steroids keep being produced, although at a lower level, in the adrenals rendered hyperplasic by the chronically increased ACTH secretion. Later, during puberty the production of C_{19}-16-oxygenated steroids increases together with that of androgens, which is a phenomenon related to adrenarche. However, C_{21}-16α-hydroxy-steroids are increased earlier, already at the age of 3–6 years and are decreased in puberty. The enhanced production of 16α-hydroxy-pregnenolone and its metabolite occurs parallel with the increasing tendency of total C_{19}-steroids and this phenomenon may infer a relationship between C_{21}-16α-hydroxylation and $C_{17,20}$-lyase activity.

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The Ca^{2+}-sensitive K^+ Transport in Inside-out Red Cell Membrane Vesicles

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Ca^{2+}-sensitive K^+ transport ("Gárdos-effect") was investigated in inside-out red cell membrane vesicles (IOVs) by measuring the effect of Ca^{2+} and other agents on the $^{86}Rb^+$ (K^+-analogue) influx. 50 μM—5 mM Ca^{2+} stimulated the $^{86}Rb^+$ influx as compared to the effect of 1 mM EDTA. Mg^{2+} and chlorpromazine inhibited the effect of Ca^{2+}, oligomycin and quinine were without effect. The permeability increase of K^+, elicited by Ca^{2+} in IOVs, corresponded to that of intact cells. The effect of Ca^{2+} was not specific to K^+ with respect to Na^+. The Ca^{2+}-sensitive K^+ channels saturated above 5—10 mM KCl in the medium. The results indicate that in IOVs the Ca^{2+}-sensitive K^+ transport has altered characteristics as compared to that of intact cells and resealed ghosts. However, in contrast to the suggestion of other investigators, it was found that some features of this phenomenon are still preserved in IOVs.

Introduction

The enhanced, selective K^+ flux following the elevation of the Ca^{2+} level at the inner side of the plasma membrane (Gárdos effect) can be consistently observed in intact red cells, resealed ghosts and in other cell types, too (Gárdos, 1958; Simons, 1976; Lew, Ferreira, 1978). Concerning the presence or absence of this phenomenon in resealed IOVs Grinstein and Rothstein (1978) reported that they could not detect Ca^{2+}-sensitive K^+ efflux in IOVs. In a recent work of Sze and Solomon (1979b) evidence was presented that Ca^{2+} stimulates the K^+ analogue $^{86}Rb^+$ influx in IOVs. However, this kind of Ca^{2+}-sensitive K^+ transport was not identical with the Gárdos effect with respect to its Ca^{2+} affinity and K^+ specificity. The Gárdos phenomenon of energy-depleted intact red cells, propranolol- or the A23187 Ca^{2+} ionophore-treated intact cells or of resealed pink ghosts shows high Ca^{2+} affinity and high K^+ specificity (Lew, Ferreira, 1978), the Ca^{2+}-dependent K^+ transport in IOVs has low Ca^{2+} affinity and shows no K^+ specificity with respect to Na^+ (Sze, Solomon, 1979b).

In this work further characterization of the Ca^{2+}-dependent K^+ transport of IOVs is presented.

Abbreviations: EDTA, ethylenediamine-tetraacetate; PMSF, phenyl-methyl-sulphonyl-fluoride; EGTA, ethylene glycol-bis (2-aminoethylether)-N-N'-tetraacetate; IOV, inside-out red cell membrane vesicle.

Materials and methods

Resealed inside-out red cell membrane vesicles were prepared and the impermeability to macromolecules and the sidedness of the vesicle preparations were determined according to the method of Steck and Kant (1974), with the modifications described by Sze and Solomon (1979a). We have diverged from the latter authors in that the membrane in the sealing medium was incubated at 37 °C for 15 min before incubation at 4 °C for 30 min, and that coaxial pestle homogenization was applied before passing the membrane through the hypodermic needle. We could obtain 75.2 ± 4.6% IOV (No: 11) without Dextran gradient centrifugation, so we did not apply this method for the separation of the resealed vesicles from unsealed membranes. For measuring the $^{86}Rb^+$ flux into IOVs, the method of filtration through millipore filter discs (Mawe, Hempling, 1965) was applied following the procedure described by Sze and Solomon (1979b). Radioactivity was measured in a liquid scintillation counter detecting the Cherenkov radiation induced by $^{86}Rb^+$.

The chemicals used were of reagent grade.

Results and discussion

Figure 1 shows that 50 μM Ca^{2+} significantly stimulates net Rb^+ entry into IOVs, and elevation of the Ca^{2+} concentration up to 5 mM causes no further increase in Rb^+ influx rate. The finding that 50 μM Ca^{2+} elicits maximal effect indicates that the $K_{M/Ca^{2+}}$ is below 50 μM. In addition, Figure 1 shows that 1.5 mM Mg^{2+} does not elicit this effect, but rather inhibits the Rb^+ influx. This finding proves that the observed effect is specific to Ca^{2+} with respect to Mg^{2+}. The degree of stimulation of Rb^+ influx by 50 μM Ca^{2+} proved to be inconsistent in the different IOV preparations, the per cent stimulation varied between 46 and 279% (100.8 ± ± 66 n = 11) at minute 6. This fluctuation resulted partly from the differences in leak permeability for Rb^+ (EDTA curves) and partly from the different rate of Ca^{2+}-sensitive Rb^+ influx. The effect of Ca^{2+} could not be stabilized by using freshly prepared IOVs for the experiments, or by applying 100 μM mercaptoethanol (SH-protective agent) and/or 200 μM PMSF (protease inhibitor) in the sealing medium. We could establish no correlation between the rate of the effect of Ca^{2+} and the IOV percentage, either. Further analysis of Figure 1 reveales that Rb^+ influx reaches a plateau between 6 and 12 minutes, and it is the initial part of the curve where the significant effect of Ca^{2+} can be observed.

Figure 2 shows that the effect of Ca^{2+} is also present when Rb^+ influx is examined in the equilibrium exchange set-up. Comparing this finding with the previous one it can be suggested that there is no need for K^+ to be present inside the vesicles for obtaining the Ca^{2+}-sensitive Rb^+ influx. Thus the mechanism of Ca^{2+}-induced Rb^+ influx in IOVs does not involve K^+ exchange diffusion.

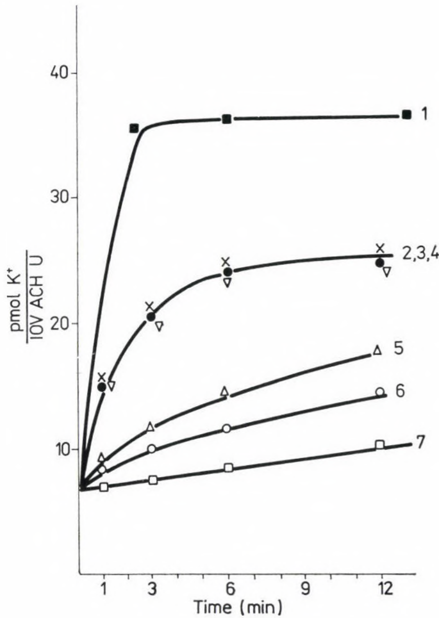

Fig. 1. Time course of net $^{86}Rb^+$ influx in IOVs. One typical experiment. The incubation medium contained 10 mM KCl, 2.5 mM Tris-HCl, pH 7.4, ~0.6 mg/ml IOV protein and Ca^{2+}, Mg^{2+}, EDTA and nigericin (K^+-ionophore), respectively. Final volume: 0.3 ml. The incubation (38 °C) was started with the addition of IOVs, and stopped by the dilution of 50 μl sample in 5 ml ice-cold quenching solution: 10 mM KCl and 2.5 mM Tris-HCl, pH 7.4. The sample was filtered through a Sartorius (cellulose-acetate) membrane disc (2.5 cm diameter, 0.45 μm pore size) and washed with 5 ml quenching solution. The radioactivity of the filter discs was determined as described in Materials and methods. IOV ACH U denotes "IOV acetylcholinesterase unit" calculated as ACH unit in the presence of 0.016% Triton X-100 minus ACH unit without Triton X-100. One ACH unit is defined as one μmol acetylthiocholine split per min, as determined spectrophotometrically, (for details see Steck and Kant, 1974). 1 = 20 μM Nigericin, 2 = 50 μM $CaCl_2$, 3 = 1 mM $CaCl_2$, 4 = 5 mM $CaCl_2$, 5 = 1.5 mM $MgCl_2$, 6 = 1 mM EDTA, 7 = 1 mM EGTA + 1.5 mM $MgCl_2$

Figure 3 shows a semilogarithmic plot of the Rb^+ flux data. The half time of 50 μM Ca^{2+}-induced Rb^+ influx is about 1.6 min, while that of the leak (EDTA) Rb^+ influx is 18 min. The calculated rate constants (k) are 26 l/h for the Ca^{2+}-induced Rb^+ influx, and 2.3 for the leak Rb^+ influx. The Ca^{2+}-induced Rb^+ influx rate constant obtained by us is higher than that obtained in intact cells by Lew and Ferreira (1976) (10 l/h) for the maximal K^+ permeability elicited by Ca^{2+}, but the permeability coefficients (roughly estimated as $P = k/A$, where P is the permeability coefficient in cm/s, k is the rate constant in l/s, and A is the specific surface area of IOVs in l/cm) are strikingly similar in IOVs and intact cells; 9.5×10^{-6} cm/s for IOVs and 1.0×10^{-7} cm/s for intact cells (Glynn, Warner, 1972). Thus the Ca^{2+}-elicited K^+ permeability in IOVs corresponds to that in intact cells.

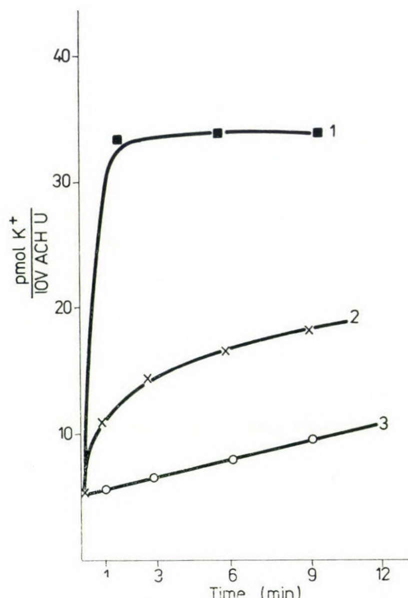

Fig. 2. Influx of $^{86}Rb^+$ in IOVs at K^+ equilibrium. IOVs were preincubated in the incubation medium for 18 h at 4 °C and incubation was started by the addition of $^{86}Rb^+$. Other conditions and procedures are the same as those described in Fig. 1. 1 = 20 μM Nigericin, 2 = 50 μM Ca^{2+}, 3 = 1 mM EDTA

The observed stimulation of Rb^+ (K^+) permeability by Ca^{2+} in IOVs is relatively smaller than that in intact cells for which three orders of magnitude stimulation has been reported (Lew, Ferreira, 1976). This finding can be explained – at least partly – by the fact that the leak K^+ permeability in IOVs exceeds that of the intact cells and ghosts. We have found k = 2.3 l/h for IOVs, and Sze and Solomon (1979a) reported k = 0.24 l/h for intact cells and resealed ghosts.

For a further characterization of the Ca^{2+}-induced channel for K^+ with respect to Na^+ was investigated. The K^+/Na^+ selectivity ratio was 2.5 ± 0.7 (3 experiments), thus the Ca^{2+}-induced channel showed a small degree of discrimination between K^+ and Na^+. This was much smaller than the K^+/Na^+ selectivity ratio of 10^3 estimated by Lew and Ferreira (1978) in intact cells. The above data confirm the observation of Sze and Solomon (1979b) that the Ca^{2+}-induced K^+ transport in IOVs does not show great specificity to K^+ with respect to Na^+.

Assaying the effects of some inhibitors of the Ca^{2+}-dependent K^+ transport in IOVs, 50 μM chlorpromazine was found to cause a 80–90% inhibition of the permeability increase for Rb^+ elicited by 50 μM Ca^{2+} (5 experiments). One tenth mg/ml oligomycin and 1 mM quinine showed no inhibitory effect (3 experiments).

In the following experiments we investigated the effects of variations of the K^+ concentration in the incubation media on the Ca^{2+}-dependent Rb^+ influx. Figure 4 shows the calculated rate constants for Rb^+ influx as plotted against the

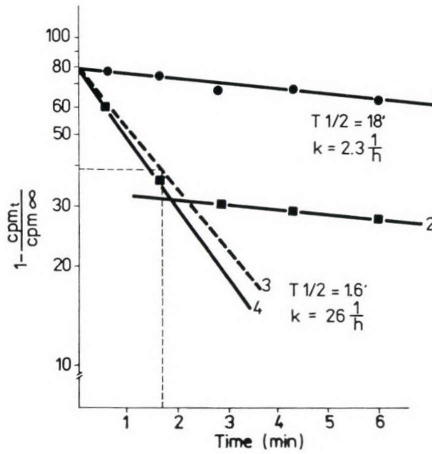

Fig. 3. Initial part of $^{86}Rb^+$ influx in IOVs. One typical experiment, cpm_t = counts per minute at time t, cpm_∞ = counts per minute in the nigericin sample. The curves were drawn and the half time ($T_{1/2}$) of $^{86}Rb^+$ influx was read from the graph. The rate constants k were calculated from $\frac{0.693}{T_{1/2}}$. 1 = 1 mM EDTA, 2.4 = 50 μM Ca^{2+}, 3 = 50 μM Ca^{2+} induced $^{86}Rb^+$ influx minus leak $^{86}Rb^+$ influx (line 4 — line 1)

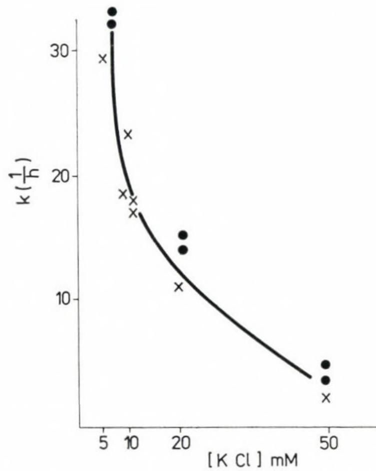

Fig. 4. The effect of K^+ concentration on the rate of net $^{86}Rb^+$ influx in IOVs, induced by 50 μM Ca^{2+}. The rate constants (k) were calculated as described in Fig. 3. ● — the osmolarity of the medium was adjusted to 200 mOsm by glucose; × — the osmolarity of the medium was not adjusted

K^+concentration. We found that the rate constants were strongly reduced above 5–10 mM KCl. Thus the Ca^{2+}-sensitive K^+ channel saturates above 5–10 mM KCl, indicating that it has only limited transport capacity. In an earlier study (Gárdos et al., 1975) it was found in our laboratory that the ^{42}K efflux from propranolol-treated ghosts does not show saturation kinetics up to 100 mM KCl. These results, together with the finding that the degree of stimulation by Ca^{2+} considerably varies, suggest that only some part of the Ca^{2+}-sensitive K^+ channels originally present in intact cells survive the IOV preparation procedure in functional integrity.

The main conclusion of the experiments described in this paper is that Ca^{2+} in micromolar concentrations elicits a maximum permeability increase for K^+ in IOVs. This observation contradicts the finding by Sze and Solomon (1979b) that the Ca^{2+} concentration initiating half maximum permeability increase of Rb^+ is about 0.7 mM. Therefore we tried to clarify why Sze and Solomon did not obtain the same effects at small Ca^{2+} concentration, as we did. We found that after Dextran gradient centrifugation the effect of 50 μM Ca^{2+} vanished.

Summarizing our findings the following statements can be made: in IOVs of the original characteristics of the Ca^{2+}-dependent K^+ transport, observed in intact cells and ghosts, the K^+ specificity and the inhibitor effect of oligomycin and quinine are lost, but the high Ca^{2+} affinity, the extent of Ca^{2+}-elicited K^+ permeability and the inhibitory effect of chlorpromazine are preserved.

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Effect of Denervation on the Properties of Actin (According to Superprecipitation Data)*

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The superprecipitation of synthetic actomyosin, formed from intact myosin and actin extracted from rabbit white skeletal muscles 14 and 75 days after denervation has been compared with that of intact synthetic actomyosin. Superprecipitation has been characterized by two parameters. 1. The value of superprecipitation ΔE determining the increase in the absorbance from minimum to maximum values; 2. the time required for the half-maximum increase ($t_{1/2}$) which is the inverse of the velocity constant. It has been shown that both the ΔE and the velocity constant decrease: the ΔE amounts to 75.4 ± 7.3 per cent (p = 0.95) and 87.9 ± 11.2 per cent (p = 0.95) after 14 and 75 days, respectively, when compared with normal; the $t_{1/2}$ increases to 41.5 ± 13.6 per cent (p = 0.95) after 14 days and 17.5 ± 11.9 per cent (p = 0.95) after 75 days. It is assumed that this effect is related to changes in the structure of actin.

Introduction

Superprecipitation of actomyosin complexes (natural or synthetic) represents a simple model which reveals some physicochemical properties of the complex components as well as their interaction. The superprecipitation method is informative also in studying proteins extracted from muscles altered pathologically.

According to the literature (Samaha, 1967; Samaha, Thies, 1973; Szöőr et al., 1977; Szabó et al., 1978) superprecipitation was studied till present time on natural actomyosin extracted from muscles in different diseases. In the present experiments the actomyosin complexes formed from myosin of intact skeletal muscles and actin of denervated muscles have been used (further on the latter one will be referred to as denervated actin).

In such a way, it seems possible to estimate the effect of denervation on the properties of actin and, particularly, its interaction with myosin during superprecipitation. Besides, the objective is to elucidate the dependence of superprecipitation on the properties of the actomyosin complex components.

Materials and methods

Rabbit hind limb was denervated by dissection and removal of a 2—3 cm part of the upper nerve trunk (n. ischiadicus). Actin was extracted according to

* This work has been made within the biophysical co-operation of CMEA.

Straub from intact and denervated white skeletal muscles (Straub, 1943). The extraction of actin was carried out at 0 °C (Drabikowski, Gergely, 1962). Myosin was obtained from the same muscles of intact animals according to Szent-Györgyi (Szent-Györgyi, 1947). Protein concentration was determined with the biuret method. In some experiments myosin kept in glycerol at −15 °C was used. Synthetic actomyosin was prepared by adding 2.5 parts of myosin to 1 part of actin (by weight). The final protein concentration in each test sample was 0.14 mg/ml. The myosin/actin ratio and the final protein concentration were the most convenient parameters to follow the time-course of the reaction. The composition of test samples for measuring superprecipitation was as follows: 7.2 mM Tris buffer of pH 7; 1.6 mM EDTA; 0.5 mM $MgCl_2$, 82 mM KCl and 0.1 mM ATP in a total volume of 10 ml. Actomyosin was obtained 14 and 75 days following denervation. The superprecipitation of the actomyosin suspension was studied turbidimetrically at 530 nm in a 2 cm glass cuvette at room temperature with a photoelectric colorimeter. After measuring the basic extinction E_0 the suspension absorbance was determined at 15 to 20 sec intervals. Before every measurement the suspension was mixed with a glass stirrer. EDTA was added to actomyosin preparation in order to slow down the superprecipitation process. The maximum absorbance increase E_{max} (extent maximum), the basic extinction E_0, the superprecipitation value $E - E_{max} - E_0$ and the time for half-maximum increase ($t_{1/2}$) were measured in order to express superprecipitation in quantitative terms.

Results and discussion

We chose experimental conditions under which clearing phase, connected with the dissociation of actomyosin was not actually observed in the preparations studied. The superprecipitation curves have a characteristic S-shape. Curve 1 in Fig. 1 illustrates the typical superprecipitation of normal synthetic actomyosin. Interaction of the denervated actin with intact myosin significantly affects the value and the rate of superprecipitation. As is seen in Fig. 1 (Curve 2), the value of superprecipitation falls to 75.4 ± 7.3 per cent ($p = 0.95$) with actin extracted on the 14th post-denervation day. With actin extracted 75 days after denervation (Fig. 1, Curve 3) the ΔE is somewhat restored and approximates its normal value. In our experiments, the ΔE constituted 87.9 ± 11.2 per cent ($p = 0.95$).

The calculations show the 41.5 ± 13.6 per cent ($p = 0.95$) increase in the $t_{1/2}$ for actomyosin containing the 14-day denervated actin, and a value as low as 17.5 ± 11.9 per cent ($p = 0.95$) for actomyosin with 75-day denervated actin. Since the $t_{1/2}$ is the inverse of the superprecipitation velocity the latter is supposed to slow down upon introduction of denervated actin into the complex.

Recent data concerning superprecipitation of natural actomyosin with different pathological alterations of skeletal and cardiac muscles (Samaha, 1967; Samaha, Thies, 1973; Szőőr et al., 1977; Szabó et al., 1978) have supplied evidence for marked changes in the character of superprecipitation and, in the first instance, a decrease in the superprecipitation value. The results of studies on superprecipi-

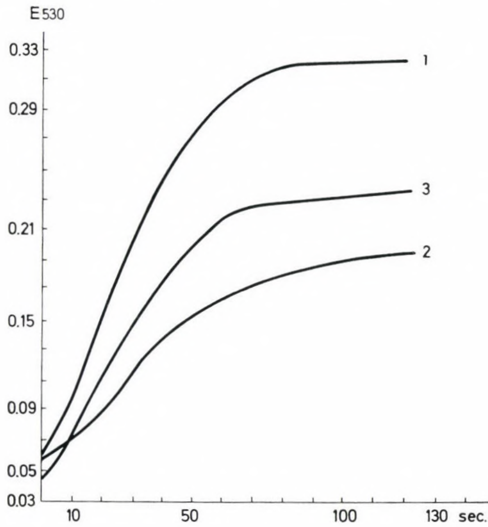

Fig. 1. Superprecipitation of synthetic actomyosin. The conditions of superprecipitation are given in "Materials and methods" 1 — normal actomyosin; 2 — normal myosin + actin after 14-day denervation; 3 — normal myosin + actin after 75-day denervation

tation following immobilization (Szöőr et al., 1977) or after removal of muscles synergists are of particular interest. Under these pathological conditions, with preserved innervation of the muscles studied, the superprecipitation value also decreases. Evidence is available for a concurrent decrease in the superprecipitation value (or the superprecipitation velocity) and Mg^{2+}-ATPase activity of actomyosin in certain dystrophies (Samaha, 1967; Samaha, Thies, 1973). These data indicate that alterations in superprecipitation are accompanied by changes in the enzymatic activity of natural actomyosin. It should be emphasized that, in the works cited above, pathologically or functionally altered muscles were used for obtaining natural actomyosin representing a complex of contractile and regulatory proteins which are most probably involved in the disturbance of superprecipitation.

In our experiments the synthetic actomyosin complex containing actin extracted from denervated muscles and myosin extracted from intact muscles was studied. This method also revealed that the protein content of the troponin–tropomyosin complex in the actomyosin studied would decrease (Drabikowski, Gergely, 1962). Since for reconstruction of the complex we used a myosin that preserved all native properties including ATPase activity, it is expedient to analyze changes in superprecipitation in relation to presumable alterations of the actin and not of the myosin.

The main conclusion of our studies is that the value and the velocity of superprecipitation of the synthetic actomyosin containing the denervated actin decrease 2 weeks following denervation; this is in full accordance with the data reported in the literature on natural actomyosin (Samaha, Thies, 1973). This phenomenon

should not be explained by the level of ATPase activity of myosin which is responsible for the quantitative characteristics of actomyosin superprecipitation. Our experiments have demonstrated that Mg^{2+}-ATPase of actomyosin does not actually vary from normal by the 14th and 75th day after denervation, a finding consistent with the idea that ATPase activity of the synthetic actomyosin depends on the properties of the myosin and not on those of actin (Katz et al., 1966).

While analyzing the obtained results one should remember that superprecipitation proceeds in at least two stages: (Samaha, 1967) formation of superprecipitation centres; (Samaha, Thies, 1973) growth of these centres (Tada, Tonomura, 1967); the first stage can proceed at a lower speed. As seen from our data, the value of $t_{1/2}$ increases. It can be assumed (and this is confirmed) by the shapes of many curves that this increase is connected with the initial phase of the superprecipitation reaction, i.e. with the deceleration of formation of the superprecipitation centres. This is probably due to a complete loss of the ability of a certain part of actin to form the actomyosin complex involved in superprecipitation.

The revealed decrease in the ΔE is evidently due to a reduction in the number of the superprecipitation centres formed as compared with normal. The superprecipitation changes can probably be explained by the presence in the complex of an actin with altered properties; in particular, with changes in the structure-forming properties of the denervated actin (Strankfeld et al., 1975) and the ability thereof to form complex with myosin (Moskalenko, Strankfeld, 1980), an event probably due to conformational changes.

This assumption is confirmed by observations of changes in the synthesis of contractile proteins after denervation (Gutmann, 1976) which intervention can result in the appearance of proteins with altered properties. This was reliably demonstrated in the case of myosin (Syrovy, 1979). The heterogeneity of actin and the existence of several forms of actin have been shown by a number of authors in the past years (Whalen et al., 1976; Vandekerckhove, Weber, 1978). The deceleration of superprecipitation revealed in our experiments is in a good agreement with the data confirming deceleration of the contraction of denervated muscles (Szabó et al., 1978).

Quantitative characteristics of superprecipitation in the case of actin extracted 75 days following denervation were partly restored which is consistent with the mode of interaction of the denervated actin with intact myosin at later points of time following denervation (Moskalenko, Strankfeld, 1980). Now, it is difficult properly to explain this phenomenon, though partial recovery of some physicochemical parameters and enzymic properties of contractile proteins at later stages of denervation is an experimentally supported fact.

The results reported here indicate that: (Samaha, 1967) Due to denervation, the complex-forming properties of actin are changed; impaired is, in particular, the actin–myosin interaction during superprecipitation. (Samaha, Thies, 1973) changes in superprecipitation of the reconstructed actomyosin might not be just the result of alterations in myosin and a decrease in the ATPase activity in particular, but they depend, to a very great extent, on the properties of actin.

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Correlation between Acetylcholine-evoked Electrical Activity, Effect of Cyclic AMP and Actual Redox State in Frog Rectus Muscle

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(Received May 20, 1980)

In view of the very high correlation coefficient between actual redox state potential level in the biophase and spike activity — triggered by acetylcholine and influenced by cAMP — in skeletal muscles it seems rather reasonable the conclusion that the redox state potential level influences the cooperative effects not only in the case of acetylcholine but, directly, also the actions of cAMP, since the type of the regulatory influence of actual redox state potential level is non-additive in some cases, though additive in others.

Introduction

Cyclic nucleotides are known to play an important part in the regulation of excitatory and inhibitory processes in nervous and muscle tissues (Shimuzu et al., 1970; Singer, Goldberg, 1970; Greengaard, Kebabian, 1974). For instance, theophylline (10^{-2} M) and caffeine (10^{-3} M) increase the response to acetylcholine (ACh) in muscles (Cole, Twarog, 1972).

Theophylline seems to cause depolarization of nerve endings but, for doing so, it seems to require prior activation of the nerve. Cyclic nucleotides have been shown to facilitate the influx of Na^+ and/or Ca^{2+} into the cells and this influx can carry depolarizing currents (Greengaard, Kebabian, 1974; Rasmussen, Tenenhouse, 1968).

From our earlier works (Puppi, Kiss, 1973; Puppi et al., 1976; Puppi et al., 1975) it is also known that both the quantitative and qualitative effects of ACh are strongly influenced by the actual redox state (E_o') in nerve, muscle and frog skin tissues. Considering that, according to Farah et al. (1969), cAMP brings about a reduction of protein disulfide groups and that, as suggested, the increase in the sodium dependent short-circuit current is related to a change in disulfide concentration and possibly also in the membrane structure (an event resulting in enhanced permeability to Na^+), it has seemed us worthwhile to investigate the assumed correlation between the effect of ACh, theophylline, imidazole and cAMP on the one hand and the actual level of redox state in skeletal muscles on the other. The significance of investigations of similar character has been emphasized by the observations of Löw et al. (1978); Lewin (1973) and Mukherjee and Lynn (1979).

According to these authors the cAMP level is significantly influenced by the actual redox state.

Materials and methods

The experiments were made on the rectus abdominis muscle of the frog *(Rana esculenta)*. The muscles excised were placed into a chamber containing Ringer solution and stretched out. Then, with the aid of a manipulator three platinum electrodes were applied to the muscles. These electrodes were insulated except for a 0.5 mm part directly connected with the muscle. Two of the electrodes served for extracellular recording of the excitatory bioelectrical activity and third one (with a sharpened tip) was inserted into the muscle. This sharpened electrode was used as a measuring electrode in the potentiometric method of E_o' measurements as described by Cater et al. (1957). The reference electrode for measurement of E_o' was Ag/AgCl immersed into the Ringer solution which the isolated organs were being kept in.

Parallel with the redox state of muscle tissue also the "spike activity" of the activated muscles was recorded with the aid of a "time-amplitude integrator". This device integrates in an electronic way into a common parameter of the frequency, amplitude and duration of all spikes occurred appearing during the excitation phase caused by ACh ("spike activity"). The amplifier by-passes electrical signs lower than 100 Hz (12 dB/octave). Time constant of the integrator circuit is 4.5 sec. The received integrated spike activity was recorded on a pen-writing potentiometric recorder and, at the same time, the under-curve area was calculated electronically on the digital output.

The biological origin of the integrated voltage was permanently controlled on an oscilloscope screen; the current was intensified by means of a controlling amplifier on the basis of sound impulses.

Theophylline, imidazole, cAMP and redox agents were administered 3 min before the application of ACh. Prior to this, control experiments were carried out with ACh only. The chemicals used were as follows: 3' 5' cyclic-adenosine-monophosphate (cAMP), 1 mM; theophylline hydrate (THEO), 1 mM; imidazole (IAZ), 10 mM; methylene blue (MB), 0.08 mM; Na-ascorbate (ASC), 0.08 mM; acetylcholine hydrochloride (ACh), 10^{-5} M. The solutions were buffered to pH 7.4 before use.

All of the computations were made with an HT–TK-891-1 type electronic calculator.

In every experimental series: n = 10.

Results

Influence of redox agents on the combined effect of cAMP + ACh

Figure 1 shows that cAMP increased both the E_o' value and the ACh spike activity significantly. Methylene blue (MB an oxidant) also increased these parameters but its effect was stronger, especially in the case of the ACh-triggered spike activity. The effect of sodium ascorbate (ASC) + ACh was smaller than the control.

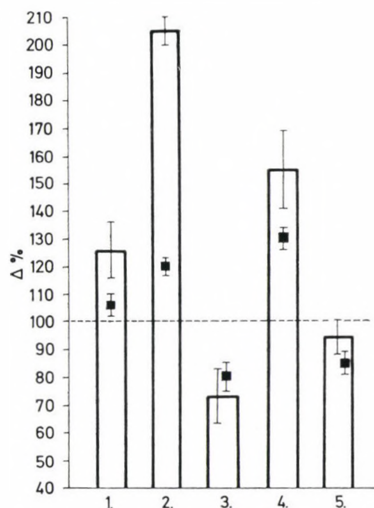

Fig. 1. Alterations in spike activity in per cent (vertical columns) and E_o' (■) values in per cent following pretreatment with cAMP + ACh (Column 1), MB + ACh (Column 2) ASC + ACh (Column 3), MB + cAMP + ACh (Column 4) and ASC + cAMP + ACh (Column 5). Control values (application of ACh only) were taken as 100 per cent. The vertical lines represent ±S.E.M.

When MB + cAMP + ACh were applied together the increase of E_o' value was the result of a strictly additive effect. This possibly means that cAMP and MB elevated E_o' through independent pathways. Contrary to these, the spike activity indicated a non-additive (competitive?) type of action between cAMP + ACh and MB + ACh.

ASC (a reductant) diminished the effect of ACh on spike activity by 27 per cent. Simultaneous application of ASC + cAMP led to an E_o' value indicative of an almost perfect additivity between ASC and cAMP but, in respect to the spike activity, the combined effect of these agents exhibited a non-additive (competitive?) character.

The correlation coefficient concerning the E_o' and spike activity changes showed a very high positivity ($r = +0.998$; $p < 0.001$).

Influence of redox agents on the combined effect of THEO + ACh

Qualitatively, the combined effect of THEO and MB was the same as that of cAMP + MB (Fig. 2). This finding indicates again that the changes caused in various parameters by the combined application of MB + cAMP involved specific cAMP mechanisms, and that the oxidizing agent influenced the effect of extra- and intracellularly increased cAMP levels to an equal extent.

On the other hand, when ASC + THEO were applied together not only quantitative but also qualitative differences could be observed depending on either the effect of extracellular application or the intracellular accumulation of

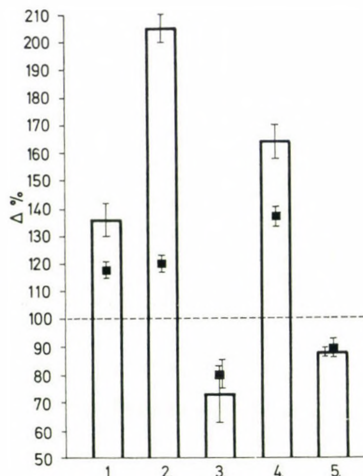

Fig. 2. Alterations in spike activity in per cent (vertical columns) and E_o' (■) values in per cent following pretreatment with THEO + ACh (Column 1), MB + ACh (Column 2), ASC + ACh (Column 3), MB + THEO + ACh (Column 4) and ASC + THEO + ACh (Column 5). The control data (application of ACh only) were taken as 100 per cent. The vertical lines represent ±S.E.M.

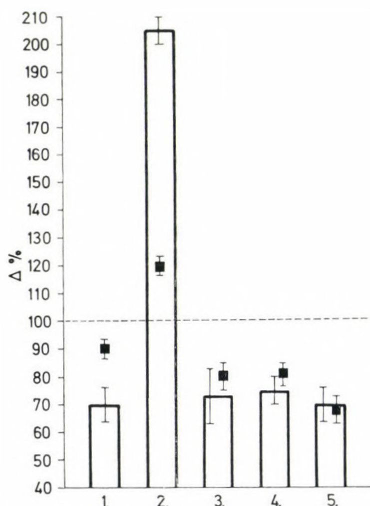

Fig. 3. Alterations in spike activity in per cent (vertical columns) and E_o' (■) values in per cent following pretreatment with IAZ + ACh (Column 1), MB + ACh (Column 2), ASC + + ACh (Column 3), MB + IAZ + ACh (Column 4) and ASC + IAZ + ACh (Column 5). The control data (application of ACh only) were taken as 100 per cent. The vertical lines represent ±S.E.M.

cAMP. As shown in Fig. 2 THEO per se evoked an increase both in E_o' and spike activity while ASC diminished these parameters significantly. In the case of simultaneous application of the two agents the E_o' level remained the same as without THEO (non-additive action), the spike activity equalled the control, as a consequence of the two antagonistic influences (additive type of effect).

The correlation coefficient concerning the E_o' and spike activity changes indicated a very high positivity also here ($r = +0.99$; $p < 0.001$).

Influence of redox agents on the combined effect of IAZ + ACh

Increasing the activity of cAMP phosphodiesterase by IAZ eventuated the following effects (Fig. 3): both the spike activity triggered by ACh and the E_o' value diminished significantly following a 3-min preincubation with IAZ. This strong action of IAZ definitively determined the spike activity levels after both MB and ASC.

None of the redox agents used was able to change the spike activity determined by IAZ; however, the correlation coefficient remained comparatively high ($r = +0.75$; $p < 0.02$).

Discussion

In view of the very high correlation coefficient existing between the actual redox state potential level in the biophase on the one hand and the spike activity (triggered by ACh and influenced by cAMP) in skeletal muscles on the other, it seems highly reasonable the conclusion that the redox state potential level influences the combined effects of not only the ACh (Puppi, Kiss, 1979; Puppi et al., 1975; Puppi et al., 1976) but, directly, also the actions of cAMP. Though cAMP has been regarded in the last years as the second messenger of ACh (Wollenberger et al., 1973; Nawrath, 1976) it is also known that ACh can provoke cAMP accumulation only through muscarinergic but not through nicotinergic receptors (Greengaard, Kebabian, 1974).

The type of this regulatory influence of the actual E_o' level is non-additive in some cases, though additive in others. The cause of these discrepancies is a question about which no acceptable hypothesis is available at present. However, the following considerations might be taken into account in this respect:

The correlation between the cAMP and ASC indicates non-additivity while additivity can be seen in the case of THEO + ASC. As known, bot THEO and ASC inhibit cAMP-phosphodiesterase (Butcher, Sutherland, 1962; Buck, Zadunaisky, 1975), while an increase in cAMP concentration will stimulate the enzyme. These differences might serve as justification. However, such an explanation does not necessarily hold true for the case of MB because this redox dye exerts a non-additive type of effect both with cAMP and THEO applications. It is, however, also known that phenthiazines inhibit cAMP-phosphodiesterase (Honda and Imamura, 1968).

Both cAMP and THEO increased the ACh triggered spike activity of skeletal muscles even though in the experiments of Dretchen et al. (1976) cAMP, in

doses up to 800 $\mu g/kg^{-1}$ had no effect on soleus muscles of the cat. This discrepancy can easily be explained considering that the cAMP concentration in Dretchen's experiments was significantly lower than in our case.

The most demonstrative documentation of our earlier assumption that E_o' level influences the ACh effects also through the cAMP system is the case of IAZ application: when the enzymatic degradation of cAMP was accelerated no redox action occurred.

Finally, the question arises whether, the effects of theophylline and imidazole are surely independent of cAMP. It is our suggestion that this possibility seems to be but slightly probable, at least in our case, because:

a) There is a striking resemblance between the action THEO and cAMP;

b) It is known that THEO (2×10^{-3} M) inhibits phosphodiesterase activity by about 90 per cent (Hackbarth et al., 1977) and, as a result, the tissue level of cAMP increases by more than 120 per cent (Dönges et al., 1977);

c) Theophylline(Dönges et al., 1977) and imidazole do not produce any contracture that could be regarded as a sign of toxicity.

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Effect of Cooling on Latency of Monosynaptic Discharges Evoked in Motoneurons of the Frog

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Cold produced increase in latency of the dorsal root evoked monosynaptic ventral root reflex was investigated in frog lumbar spinal cord. Attempts were made to separate changes in the synaptic transmission time from changes in the conduction velocity of axons involved in the reflex pathway. Reduction of the temperature by 10 °C from the initial values of about 17—19 °C produced a 50—60 per cent increase in reflex latency, similar to the degree of slowing down of the axonal conduction velocity. The finding fails to conclusively support the chemical synaptic transmission mechanism of the ventral root discharges studied and calls for more detailed investigation of the phenomenon.

Introduction

Recent experimental findings provide strong evidence for the electrotonic mechanism of the dorsal root (DR) evoked excitatory postsynaptic potentials (DR-EPSPs) in the frog motoneurons (Shapovalov, Shiriaev, 1978; Shapovalov et al., 1978; Alvarez-Leefmans et al., 1979). Previous data (see Simpson, 1976) have shown that transmission from DR fibres to motoneurons can also be chemical. One should therefore consider either separate groups of primary afferents to transmit chemically and electrotonically, respectively, to the motoneurons, or the existence of a dual (chemical and electrotonic) mode of transmission in some endings of DR fibres. Dual mode of synaptic transmission has first been demonstrated in the avian ciliary ganglion (Martin, Pilar, 1963); the most convincing evidence for such transmission has been obtained from the spinal cord of sea lamprey (Rovainen, 1974; Ringham, 1975; Martin, Ringham, 1975). Many other examples are also known from various systems (see Korn, Faber, 1979). However, it has never been tested wether the monosynaptic discharges resulted from DR–EPSPs in the frog motoneurons are due to chemical or electrotonic transmission.

Dependence of synaptic delay on the temperature is a widely known consequence of chemical processes involved in release and binding of transmitter substances in junctions operating chemically. In electrotonically coupled systems no similar dependence on temperature is expected. Investigation of temperature dependence of monosynaptic reflexes in the frog offers, therefore, a way of testing the chemical versus electrotonic mechanism of postsynaptic potentials from which

the discharges recorded in the ventral root (VR) axons take their origin. Advantage of this rather easy approach is its allowing the direct measurement of the output component of the reflex in a large number of motoneurons but the technique of electrophysiological analysis of DR–VR reflexes has inherent inaccuracies which also are to be taken into consideration.

Materials and methods

The experiments were performed in adult frogs *(Rana esculenta)*. Dissection and maintenance of spinal cord in situ were described in detail elsewhere (Czéh, Székely, 1971; Czéh, 1979). In short, DR fibres were stimulated electrically with 0.1 ms 1 – 2 V rectangular impulses and the evoked discharges of the motoneurons recorded with bipolar platinum electrodes on the VR axons. Distance between stimulating and recording electrodes via the spinal cord was 2 – 5 cm in all cases. Afferent volleys were monitored monopolarly at the entry zone of the DR stimulated. Conduction time in the motor axons was calculated by making use of the records of the antidromic focal potential in the ventral horn. The amplified signals appearing on the oscilloscope were photographed.

Body temperature was controlled by changing the temperature of a water bath in which the frogs were kept during the experiments. Ice blocks were used to cool the water and periods of at least 20 minutes were allowed for stabilization of body temperature after stabilization of the water temperature. However, part of the dorsal surface of the head and body, where the spinal cord and the nerves were dissected and mounted on electrodes had necessarily to be kept out ouf water and exposed to the room temperature. We realized that this technique was insufficient to maintain body temperature exactly at the level of bath temperature, especially when it had to be maintained at 8 – 10 °C in a room of 25 – 30 °C. Body temperature was measured by a small thermistor inserted the back musculature above the surface of the water bath. Occasional differences in local temperature of the spinal cord itself and of the muscles were not measured because we did not want to disturb the reflex transmission by application of any device to the segments investigated.

Results

Several pilot experiments were made to find a way to eliminate as many disturbing factors as possible. One of the most important questions was to establish the temperature range within which neither irreversible changes nor any unknown alterations in the refiex mechanism would prevent us from drawing conclusions. The rate and direction of artificial change in temperature had also to be determined. Monosynaptic discharges in the frog motoneurons are known to be heavily influenced by factors like general condition of the animal, dosage and type of drugs

given as well as the values of temperature kept constant for long periods. We restricted our work for studying animals which met the following criteria: 1. Fast rising, large amplitude VR response with segmental latency of less than 4.0 ms at 17–18 °C. This criterion indicated how the monosynaptic reflex survived treatments involved how the monosynaptic reflex survived tratments involved in preparing the frogs for the experiment; at the same time it allowed to link the present results to those obtained previously in this range of temperature (see Czéh, 1979). 2. Fast and continuous blood circulation in the small vessels seen on the dorsal surface of the spinal segment studied. Slow or interrupted blood circulation in these vessels was considered as early sign of deterioration and frogs with poor circulation were rejected. 3. Flexor reflex of good motility in the forelimbs upon stimulations such as mechanical pression of a finger. Since hindlimbs had to be denervated, general excitability of the spinal cord could be checked by such an adequate stimulation of the forelimbs. 4. All the above criteria had to survive about 10 °C decrease in temperature with alterations no other than those reasonably attributable to cooling. Practical considerations suggested restriction to a single slow cooling process in each experiment, since rewarming and repeated cooling within short periods of time invariably caused deterioration in some of the above criteria. Very slow rewarming and repetition of slow cooling would expand the experiment over 10 hours, when persistence of the initial conditions had already become questionable.

Changes in latencies of the evoked responses were studied systematically in 5 frogs which met the above criteria throughout the cooling process and showed sufficient recovery afterwards. In these animals the afferent volley in the DR fibres and the efferent response in the motor axons were recorded on oscilloscope and photographed first at 17–20 °C. Pictures were also taken several times during slow cooling to 6–10 °C. Care was taken to allow stabilization of the temperature before taking pictures at each level. In addition, the conduction time of VR axons was also determined by means of the antidromic volley technique.

Figure 1 a shows typical responses evoked by stimulation of the tenth DR and recorded from the sciatic nerve at 18 °C. The bottom trace is a response produced by the same stimulation at a point on the dorsal surface of the spinal cord between the ninth and tenth DR. Latencies of these responses to a similar DR impulse clearly increased with cooling to 9.8 °C (Fig. 1b). Of course, not only the DR but also the VR axons suffered some reduction in their conduction velocity, as indicated by the increase in latency of the field potential responses initiated by antidromic invasion of the ventral horn motoneurons (Figs 1c and 1d). In Fig. 1e changes in latency of the afferent volley and the motor response are plotted against temperature as derived from two experiments. Specimen records from one of them is shown in Fig. 1a and Fig. 1b. In the remaining three experiments, the responses were sampled at the extreme values and only a few intermediate values. Data of these experiments would give similar but less detailed plot as shown in Fig. 1e. Furthermore, rate of change in conduction velocity of motor axons was found to be parallel with that of the DR fibres.

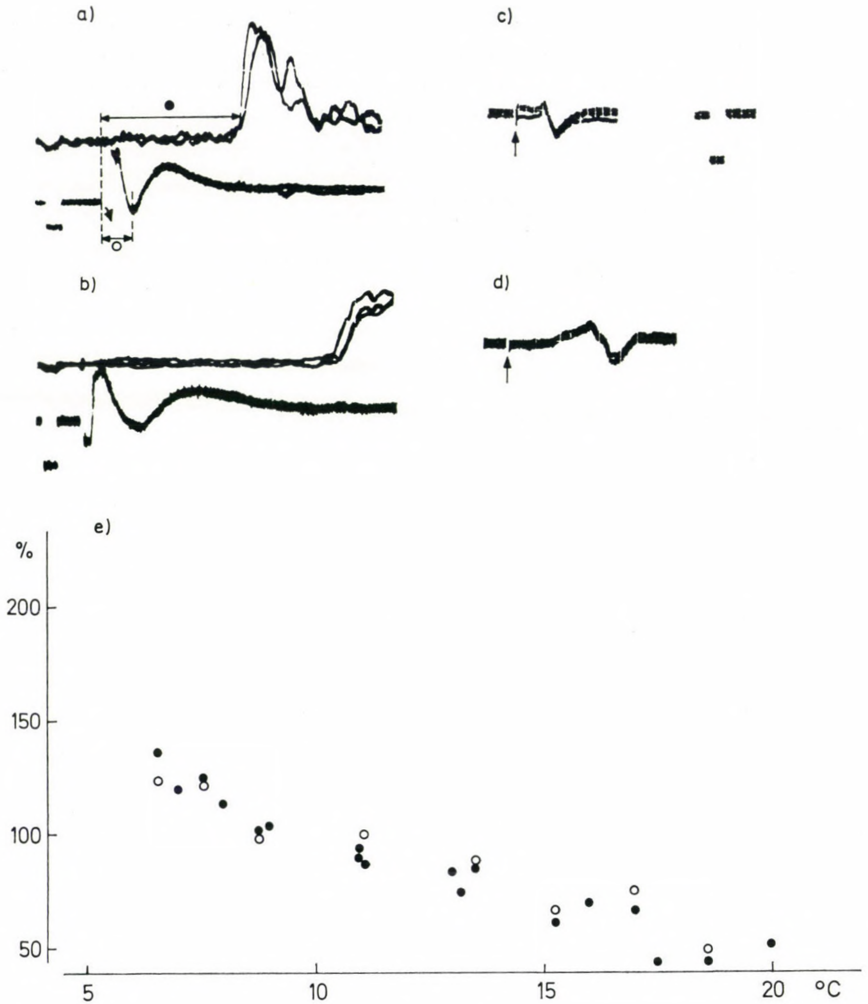

Fig. 1. Effects of cooling on latency of responses evoked in frog spinal cord. a: Upper traces show superimposed records of DR evoked VR reflex at 18 °C. Bottom traces: simultaneously photographed records of the afferent volley in the DR fibers. Distance marked with a dot shows the latency of the VR reflex and that with an open circle illustrates our technique of measurement of the latency of DR volleys. b: Same as in A, but after stabilization of the body temperature at 9.8 °C. c and d: Same frog, antidromic field potential responses in the ventral horn evoked by stimulation with the electrodes used for recording the VR reflex in a and b. c at 10 °C and d at 9.8 °C. The 1 ms 0.5 mV calibrating impulse in c applies for the other records as well. e: VR reflex latency (dots) and afferent volley latency (open circles) plotted against temperature. The data are the results of two experiments, one of them is also shown in a and b. Individual values are normalized by taking the latencies measured at 10 °C for 100 per cent

The conclusion of these experiments is clear: decrease in conduction velocity of the peripheral nerves and increase in overall latency of the evoked reflex response have identical rate of change during cooling. Calculation of central delay of the synaptic transmission from these data would result in a similar increase rate with decreasing temperature.

Discussion

To the best of our knowledge no data have been published on measurement of the temperature dependence of the monosynaptic DR-VR reflex in the frog spinal cord in situ. Tebécis and Phillis (1968) measured the cooling-induced change in latency of DR–VR discharges on isolated spinal cord of *Bufo marinus*. They attributed the extent of the increase found to the well known decrease in conduction velocity of fibres involved in the reflex pathway (Tasaki, Fujita, 1948; Frankenhaeuser, Moore, 1963; Nagy et al., 1978). Therefore, the conclusion of Tebécis and Phillis (1968) has been corroborated by our results: synaptic delay itself is no more affected by changes in temperature than is the conductive properties of the fibres.

However, the conclusion that the apparent lack of central effect of temperature rests upon several assumptions which must be the subject of further experimental work. First of all, synaptic transmission should be analyzed by intracellular recording technique, a work being now in progress in this laboratory. Secondly, effects of cooling synaptic transmission in the frog have to be studied also in other types of central synapses, for example in synapses of descending pathways on the motoneurons.

The most important question to be answered is whether the mechanism of discharges will be modified by cooling. One may assume that, at high temperature, the chemically transmitting synapses dominate the generation of action potentials of the motoneurons while at low temperature, where the chemical processes should be much slower, the relative importance of electrotonic coupling may come in foreground. If this assumption is right then the relationship in Fig. 1e should be considered incorrect. Alternatively, one might propose that the synaptic transmission is chemical throughout the temperature ranges studied but has, for some unknown reasons, low sensitivity to cooling. This assumption would not contradict the results of earlier work of others (Shapovalov, Shiriaev, 1978; Shapovalov et al., 1978; Alvarez-Leefmans et al., 1979) who have found the purely electrotonic DR–EPSPs to be rather small in amplitude. Conflicts would arise, however, from comparisons with results of another work that have demonstrated a considerable increase in latency of chemical synaptic delay in the central nervous system of the leech (Nicholls, Purves, 1972). As long as the above questions remain unsettled, one can evaluate our finding as a support for the electrotonic origin of the VR discharges. In other words, depolarization arising from activation of the electro tonic couplings between primary afferents and motoneurons would be the primary-cause of the firing of the motoneurons. At higher temperatures, discharges gener-

ated through chemical synapses may occur at about the same time but at low temperatures they would either be lacking or merge into the polysynaptic components of the VR response. Monosynaptic discharge in some motoneurons could recruit many other cells into the firing group through electrotonic interactions known to operate among the motoneurons in the frog (Washizu, 1960; Magherini et al., 1976; Sonnhof et al., 1977; Shapovalov, Shiriaev, 1978).

An entirely different assumption may be deduced from a theory which considers biological excitatory processes as coming from certain semiconductor-like properties of the excitable tissue (Ernst, 1956, 1963; Lakatos, 1962; Lakatos, Kollár-Mórocz, 1966, 1967, 1969a,b; Lakatos, 1967, 1975; Nagy et al., 1978; Tigyi, Lakatos, 1978).

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Contractile Properties of Glycerol-treated Muscle Fibres Prepared by Rome's Procedure*

(Short Communication)

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Glycerinated muscle fibre preparations were first made by Szent-Györgyi in 1949. In his investigations rabbit psoas muscles were treated in 50 per cent glycerol solution which, within 3—4 weeks, destroys and removes the sarcolemma, the sarcoplasmic reticular system, the mitochondria and other cell organelles. The structure of myofibrils is identical with the living muscle; thus, the glycerinated myofibril can be regarded as an organized actin-myosin-tropomyosin-troponin system permeable to ATP, cations and anions. The classic method of Szent-Györgyi (1949) was applied by a number of authors either without (Helander, 1962; Ranney, 1954; Yoshida, Tawada, 1976) or with some modification (Weber, 1951; Lee, 1961; Watanabe et al., 1964; Sexton, 1967; Kalamkarova et al., 1968; Borejdo, Oplatka, 1976; Cooke, Bialek, 1979). It has been the aim of those modifications to assure good reproducibility of the ATP-Ca^{++}-induced contractions by the use of various buffers. Despite the modifications the only disadvantageous property of the original method has not yet been eliminated: i.e. destruction of the membrane is a slow process lasting 3—4 weeks. Rome (1967, 1972) performed X-ray diffraction studies and for this purpose, destroyed the membranes by osmotic treatment that accelerated the process of membrane destruction. It is, however, doubtful whether the fibre preparation can retain its contractile ability. It is the aim of our present experiments to try and give an answer to these questions.

New Zealand white rabbits of 2.5—3 kg body weight were bled after one day fasting, then pelted and cooled. Fibre bundles of psoas, gastrocnemius, soleus and extensor digitorum longus (EDL) muscles of 2—3 cm length and 2 - 3 mm in diameter were excized and tied to wooden sticks, taking great care that the muscles keep their original length. The fibres prepared in this way were incubated according to Rome's procedure in 50 per cent glycerol solution containing 6.67 mM phosphate buffer at 0 °C (pH 7.0). After 30 min the fibre bundles were transferred to a slightly hypotonic salt solution (100 mM KCl, 1 mg $MgCl_2$, 6.67 mM phosphate buffer, pH 7.0). Then the exchange of the glycerol solution and salt solution was repeated twice at 30 min intervals. Following this three-hour procedure the fibres were kept in 50 per cent glycerol solution at 0 °C for 24 hrs. Then the preparation

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was transferred to fresh 50 per cent glycerol solution and stored at −20 °C until required. As a result of pretreatment, the preparations obtained in this way can be used for experiments as early as 48 hr.

Prior to use the fibre bundles were immersed in 10 per cent glycerol of 4 °C for 15−20 min. Subsequently, for the finer preparation of the fibres, various methods were tested and the following one was found to be the most useful. Under preparative microscope the fibre bundles were placed into a *pre-relaxion solution* A containing 100 mM KCl, 5 mM $MgCl_2$, 4 mM EGTA and 10 mM histidine (pH 7.0) and fibres 0.2−0.4 mm in diameter were prepared. These fibres were fixed to the arms of the differential capacitance transducer and incubated in the pre-relaxation solution for 2−3 min. Then the pre-relaxation solution was replaced by a *relaxation solution* B containing also ATP and having the composition as follows: 100 mM KCl, 5 mM $MgCl_2$, 4 mM EGTA, 5 mM ATP and 10 mM histidine (pH 7.0).

After relaxation of the fibre the relaxation solution was replaced by a *relaxation-washing solution* C of lower EGTA but identical ATP content (100 mM KCl, 5 mM $MgCl_2$, 0.15 mM EGTA, 5 mM ATP, 10 mM histidine, pH 7.0). After 0.5−1 min contraction was initiated by a *contraction solution* D containing 100 mM KCl, 5 mM $MgCl_2$, 0.15 mM EGTA, 5 mM ATP, 0.15 mM $CaCl_2$, 10 mM histidine; pH 7.0) pCa was calculated according to Bremmel and Weber (1975). When tension reached its maximum the solution D was changed for solution B, an intervention resulting in a quick relaxation of the fibre. After washing out the relaxant, contraction could be elicited again.

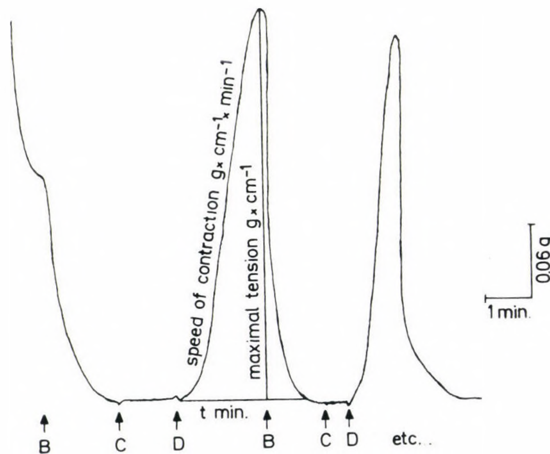

Fig. 1. *Isometric tension of rabbit psoas muscle fibres*. Fibres were first bathed in relaxation solution B. After relaxation the C relaxation washing solution was replaced. The contraction was iniciated by contraction solution D (see text)

The isometric changes were recorded with a capacitance transducer that gave linear responses in the 0–2.5 g range. The diameters of the fibres were measured with a microscope at several positions along the fibre and the values obtained averaged. The values of tension and velocities were referred to 1 cm circumference.

Table 1 summarizes the ATP-Ca^{++}-induced tension values and velocities of the different fibre preparations.

Table 1

ATP-induced isometric tension of glycerol extracted fibres from different rabbit muscles

	Maximal tension $g \times cm^{-1}$	Speed of contraction $g \times cm^{-1} \times min^{-1}$
m. soleus	3.99±0.82 (n = 8)	0.91±0.16 (n = 8)
m. gastr.	6.24±1.55 (n = 15)	1.84±0.52 (n = 16)
m. EDL	2.08±0.64 (n = 16)	0.43±0.12 (n = 16)
m. psoas	7.60±0.66 (n = 32)	2.68±0.35 (n = 32)

On the basis of the investigations presented we have drawn the conclusion that Rome's method does not impair the contractile system, the fibrils retain their contractility, and the glycerinated fibres prepared with such pretreatment can be advantageously employed in investigations on contractile properties.

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Conformation-Activity Relationships of Corticotropin Segments*

(Short Communication)

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It is known that polypeptide hormones are produced by enzymatic degradation of high molecular precursor proteins during the maturation process in the hormone secreting cells. In general, their structuration is lower than that of the precursors, owing to the smaller molecular size. In contrast to proteins, the side chains responsible for receptor binding and biological activity, are located mainly adjacent to one another in the peptide chain forming binding and active centers.

It is generally accepted that in the corticotropin molecule, which consists of 39 amino acid residues, the sequence 15—19 is responsible for the binding, and residues 4—9 are responsible for eliciting the hormonal response (Fig. 1), therefore several of the shorter segments of the native hormone exhibit hormonal activity (Schwyzer, 1977).

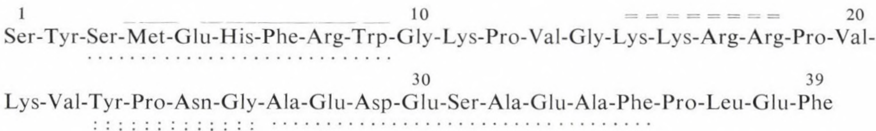

Fig. 1. Amino acid sequence of human corticotropin. ——— active site; === binding site; ······ α-helix forming potential; : : : : : β-turn forming potential

On the other hand, it is also well known that the initial step in the hormonal action is an interaction between the hormone molecule and a special receptor protein on the surface of the target cells. In the formation of this hormone-receptor complex the conformational fitness of the hormone molecule plays a decisive role, which depends also on structural factors other than the binding and active sites.

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Based on these considerations, recently we have studied the conformational properties of the corticotropin molecule by different spectroscopical methods. According to earlier spectroscopic data, the hormone has been considered to have a completely random structure in aqueous solution (see in Greff et al., 1976 refs. 3–12). However our calculations made according to Chou and Fasman (1974) indicated tendencies of the molecule to form secondary structures (Lőw et al., 1975). The regions 3–9 and 27–35 may adopt an α-helix, and the residues 23–26 are very favorable for a β-turn formation (Fig. 1).

The helicophilic character of the molecule has been substantiated by CD spectroscopic measurements (Greff et al., 1976). Though corticotropin has a "random" spectrum in aqueous solution, in trifluoroethanol characteristic helix-type spectra are obtained. Evaluation of the CD spectra of the shorter segments clearly indicates definite proneness to helix formation at the N-terminal part of the molecule. From the effects of pH on the CD and $^{1}H-NMR$ spectra it can be concluded that the N-terminal part of the molecule has an organized structure even in water (Toma et al., 1976). In addition, IR measurements have shown six slowly exchanging protons in the molecule, indicating the presence of hydrogen bonds characteristic of helix-like arrangements (Nabedryk-Viala et al., 1978). Thus the spectroscopical data strongly support the idea of a helical organization of the N-terminal part of corticotropin even in aqueous solution.

The presence of a β-bend at sequence 23–26 has been suggested by the chemical shifts of relevant signals in the ^{1}H- and $^{13}C-NMR$ spectra of the various segments (Toma et al., 1976, 1978, 1981). *Cis-trans* isomerism of the X-Pro bond, has been observed only in the environment of Pro^{24} and failed to appear in the case of Pro^{12} and Pro^{19}; i.e. characteristic signals are found for the aromatic protons of Tyr^{23} and the methyl groups of Val^{22} in $^{1}H-NMR$ spectra, and for the majority of the carbon atoms of Tyr^{23}, Pro^{24}, Asn^{25} and Ala^{27} in $^{13}C-NMR$ spectra. The splitting of these signals clearly indicates the big difference in the spatial arrangement of the side chains in the *cis* and *trans* conformers, and reflects a non-random conformation in sequence 22–27. In agreement with the prediction of Chou–Fasman calculations, we suppose that the observed rigidity in the environment of Pro^{24} is due to formation of the β-bend.

Spectroscopical data therefore unequivocally supports the idea that the corticotropin molecule has, even in water, a non-random conformation. It is very likely that the sequence 3–9 adopts an α-helix conformation and sequence 23–26 forms a β-bend. In trifluoroethanol, which may better resemble the natural environment on receptor proteins, the helix content is much higher than in water.

The next question is whether the conformational properties of corticotropin discussed above are in accordance with the biological properties or not? It is known, e.g., that the biological half life of a shorter segment of corticotropin is about one half or one third as compared with the intact hormone. It seems that the C-terminal sequence, missing in the shorter segments, is capable of protecting – at least partly – the molecule from metabolic degradation (Schwyzer, 1977). The protective effect may now be explained by the presence of the β-bend discussed

above, which may bring about the steric proximity of the C-terminal part and the sensitive basic core (segments 15–19) of the peptide chain.

In some cases the differences observed between the biological activities of some corticotropin segments may also be interpreted by the different conformational properties which depend on the chain length. For instance, Lowry and McMartin found that on isolated adrenal cells the segment 1–24 was sevenfold more potent than the intact hormone (Lowry, McMartin, 1974). This fact may be explained again by the effect of the C-terminal sequence which, when folding back by the β-turn can hinder not only the proteolysis of the molecule, but also the formation of the hormone-receptor complex.

In our biological experiments we have compared the activities of segment 1–24, segment 1–32 and the intact hormone, under *in vitro* and *in vivo* conditions. On isolated adrenal cells the two segments proved to be more potent. The relative molar potencies of corticotropin 1–39, 1–32 and 1–24 were 100, 145 and 158 %, respectively. In contrast, the higher *in vivo* steroidogenetic effects were observed in the case of the two longer peptides. The lowest plasma corticosterone levels were found at each dose in animals treated with segment 1–24 (Table 1).

Table 1

Effect of intravenous injection of various corticotropin segments on plasma corticosterone level of hypophysectomized rats

Corticosterone was determined by fluorimetric method (Guillemin et al. 1958). Male rats were treated 2–4 hours after transauricular hypophysectomy

Corticotropin+	1–39			1–32			1–24		
Dose, pmol/kg	1	3	10	1	3	10	1	3	10
Time, min	Plasma corticosterone nmnl/l								
0	60	60	60	60	60	60	60	60	60
10	210	540	700	300	480	610	120	190	550
20	150	190	470	120	230	360	100	140	330
30	70	90	340	60	120	340	60	150	290
60	60	60	80	60	60	80	60	60	60

+ For numbering see Fig. 1

Considering that in the *in vitro* experiments mostly the hormone-receptor interactions are expressed, whereas the *in vivo* results are strongly influenced by metabolic effect, it can be concluded that the shorter C-terminal part in sequence 1–32 disturbs the receptor interaction less than in the case of the intact hormone, but it gives a satisfactory protection against proteolytic attack. This may be the reason why the segment 1–32 exhibits an extremely high activity in the ascorbic acid depletion test (190 IU/mg peptide), higher measured so far on any other non-modified segments of corticotropin. It seems therefore that the segment 1–32 advant-

ageously combines the high intrinsic activity of shorter segments and the greater metabolic stability of the intact hormone.

The main conclusion of our investigations is that in the study of structure-activity relationships conformational properties must be considered even in the case of molecules considered as flexible as peptide hormones.

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Book Reviews

Membrane Fluidity: Biophysical Techniques and Cellular Regulation by Morris Kates and Arnis Kuksis, Humana Press Inc., Clifton, New Jersey, 1980, 464 pages.

The present volume is based on the proceedings of a symposium on "Control of Membrane Fluidity" held in assiciation with the 11th International Congress of Biochemistry, Toronto, 1979.

The book makes an effort to review much of the exciting current research on cell membrane fluidity. Investigation of this complex property of membranes draws together many different areas, and tries to decide the role of lipids in controlling the structural and functional properties of the cell membrane. The main problems are discussed in five sections:

I. Measurement of membrane lipid composition and fluidity. The articles deal with recent methodological advances in the determination of the composition of membrane lipids, that are now coming into use in this field. These new biophysical techniques are capillary chromatography, and selected ion mass spectrometry, which were found very useful to determine complex molecular species at the microgram level. An improved densitometry employing high performance TLC plates is also discussed. New approaches have been made in membrane lipid analyses by differentiation between the inner and outer halves of the bilayer, as well as among various domains of either of the two monolayers. These new techniques are extremely important, since calculation of membrane fluidity without prior detailed knowledge of submembrane distribution of the component lipid classes is impossible. This section concludes with a brief examination of the physico-chemical meaning of the term fluidity.

II. Correlation of membrane fluidity with physiological activity. These papers describe various correlations of changes in the physiological activity of the system. Normal or inverse correlations are recognized between membrane fluidity and certain enzymic activities, and the correlations found are of considerable importance for understanding a variety of enzyme reactions. But the question remains open, whether phospholipides merely provide a general matrix capable of organizing enzyme activities, or are there some special lipids to determine enzyme reactions.

III. Fatty acid changes accompanying physiological events. This section describes those changes in the fatty acid composition of cell membranes that accompany specific physiological events. Among others the importance of phospholipid desaturase enzyme controlling membrane fluidity in rat liver microsomes is discussed, and results are presented about the interaction of the bovine brain phospholipid exchange protein with bilayer liposomes of various lipid content. Detailed examination about the regulation of membrane fluidity in prokaryotic organisms are reported. Results indicate that homoviscous adaptation in yeasts need not be tightly regulated, and changes in the lipid phase state (e.g. a shift from all liquid-crystalline lipids to a gel-liquid-crystalline mixture) can affect certain membrane functions as well.

IV. Phospholipid changes accompanying physiological events. Although the relationship between the phospholipid head group

composition and membrane fluidity has not yet been as extensively investigated as that between fatty acid composition and membrane fluidity, there is good evidence that certain reciprocal changes may be involved. An interesting study deals with the increased turnover of phosphatidyl inositol in response to activation of various types of plasma membrane receptors, the so called "phosphatidylinositol effect". Conversion of phosphatidylinositol to diacylglycerol probably increases membrane fluidity, and contributes to the fusion of transmitter vesicle membrane and nerve ending plasma membrane for release of transmitter. Another interesting report deals with ligand-induced clustering of glycosphingolipids in lymphocytes. This "patching and capping" is one of the earliest observable phenomena following ligand binding. Gangliosides readily form micelles, and it is possible that their clustering is associated with the Ca^{++} influx of cells. Assessment of the functional role of the polar head group portion of membrane phospholipids *in vivo* is a difficult task. Therefore the study about the *in vivo* modification of phospholipid polar head groups using a lipid analog precursor is of particular interest. This choline analog interferes with cholin metabolism, and moreover causes highly specific changes in selected membrane enzyme activities.

V. Homeostatic regulation of membrane fluidity. Microorganisms are particularly useful for studying the control of membrane fluidity for two reasons. Firstly, they respond readily to changes in their enviroment, secondly it is relatively easy to isolate mutants, which provide a great deal of information. The long established observation in microorganisms of an increase in fatty acid unsaturation with decreasing growth temperature was well illustrated by the results obtained with an aerobic bacterium. Furthermore for the same growth temperature, the phospholipids isolated from the different thermal categories of yeasts showed a similar unsaturation index. The regulation of membrane fluidity in eukaryotic cells involves two separate but coordinated phenomena: the enzymic alteration of membrane lipids, and dissemination of the newly altered lipids to the various distinct membranes throughout the cell. Tetrahymena pyriformis proved to be a useful model system for a better understanding at the interrelationship between lipid composition, physical states and function of the eukaryotic membrane.

The detailed investigations clearly indicate that membrane fluidity is a complex and variable phenomenon which can be influenced by all factors affecting the structural and/or functional properties of cell membrane. A great value of the volume is the general recognition that the idea of membrane fluidity requires a revision of its working definition, or even a full replacement by some other more suitable concept.

KATALIN BARTHA

Nucleoside Analogues. Chemistry, Biology and Medical Applications. Edited by Richard T. Walker, Erik De Clercq and Fritz Eckstein. Plenum Press, New York, 1979.

This book contains the fifteen Review Lectures given at a joint NATO Advanced Study Institute and a FEBS Advanced Study Course, held at Sogesta, Italy from May 7 to May 18, 1979. As the editors have stated in the Preface of the book, "the aim of this meeting was to gather together many of the experts in the different scientific disciplines which are involved in the design, synthesis, testing and clinical use of nucleoside analogues, primarily as anti-viral and anti-cancer agents, and to discuss in depth the fundamental principles of each discipline so that participants could understand each other's problems and to be more aware of the information required and that which can be obtained". The attainment of this aim is guaranteed by the reviewers, all of them being internationally renowned specialists of their own topics.

Six of the fifteen reviews deal with the chemistry of nucleotide analogues. W. Saenger and S. S. Danyluk elegantly summarize the conformational properties of nucleosides and nucleotides and the use of X-ray crystallography and NMR spectroscopy in the determination of conformation. In H. Vorbrüggen's paper on the chemical methods for nucleoside synthesis, emphasis is placed on the modified silyl-Hilbert-Johnson reaction. This wonderful paper clearly shows how a chemical problem should be presented to become easily understandable also for non-

chemists. The papers by M. J. Robins and L. B. Townsend deal with the chemical transformations of the heterocyclic bases of pyrimidine and purine nucleosides, respectively. The admirable review of J. G. Moffatt on the chemical transformations of the sugar moiety of nucleosides may be regarded as the most complete review available so far.

Three papers are concerned with the biochemistry of nucleoside analogues. S. S. Cohen reviews the action of aranucleosides and aranucleotides on cellular and viral multiplication. The papers by W. E. G. Müller, as well as those by W. H. Prusoff and P. H. Fischer deal with the metabolism, antiviral and antitumour activity of purine and pyrimidine nucleoside analogues.

The remaining six papers relate to the testing and clinical use of nucleoside analogues. R. W. Sidwell and F. M. Schnabel jr. summarize the test systems for evaluating the antiviral and antitumour activity of nucleoside analogues. Y. C. Cheng, M. Ostrander, D. Derse and J. Y. Chen describe the development of antiherpes virus agent. T. C. Merigan and H. E. Renis review the work on antiviral chemotherapy with particular emphasis on the attractive possibility of combining chemo- and immunotherapy. A clear summary has been prepared by A. Rossi on the clinical use of nucleoside analogues in malignant disease. The last short paper by S. Schultz deals with the industrial development of new drugs.

This book provides a good outlook on the whole complex field of nucleoside analogues. It will be of great value to both clinicians and researchers.

J. THOMASZ

Photoreception and Sensory Transduction in Aneural Organisms by F. Lenci and G. Colombetti (eds), NATO Advanced Study Institutes series, Plenum Press New York, London, 1980.

Photosensory behaviour is one of the most interesting features of life, ranging from the simplest photokinetic response of certain microorganisms to the vision of vertebrates. There is a great diversity in the mechanism of photoreception, in the transformation of stimulus as well as in the nature of response, but what is perhaps more surprising, there are also a number of common features.

The advantages of microorganisms for research on sensory responses are clear. The whole process from stimulus to response occurs in a single cell, and these cells are usually quite accessible to biochemical and genetical studies.

Light is one of the most appropriate stimuli to study sensory behaviour. The energy, intensity and in some cases even the location of the stimulus can be easily controlled. Short light pulses enable us to induce single turnovers of the reaction chains. Since the different sensory mechanisms for different stimuli are similar and in some cases almost the same, research in photoreception is very important.

This book collects the lectures on this topic, held during a high-level course organized by the NATO Advanced Study Institute in Italy, September 3—14, 1979.

After an overview on sensory transduction in aneural organisms and a brief introduction to optics, the first part of the book presents a phenomenological description of the different photomotile responses. The discussion of the photomovements of flagellates, using several examples that show diverse response "strategies", is followed by an article on photomotions of gliding organisms and bacteria. The phototropism of lower plants, especially that of single fungal cells is dealt with as a typical blue light effect.

An important section on methodological questions and difficulties of measuring photoresponses concludes this part of the book.

One of the most interesting papers presented is that on the photoreceptive apparatus of flagellated algal cells. The author (P. Omodeo) treats the morphology of photoreceptors and compares the cybernetic models for self-directing systems to the anatomical parts of the cells. In a study of the localization and orientation of different photoreceptor pigments, such as chlorophyll, rhodopsin, bacteriorhodopsin, phytochrome and cryptochrome, which show a great diversity in structure and function, it is demonstrated that a photoreceptor pigment is necessarily associated with a biomembrane.

After a discussion of methods of identification of photoreceptor pigments, a chapter, introducing the next part of the book, sup-

plies a theoretical background to primary photophysical and photochemical reactions. The candidates for blue light photoreception, flavoproteins and carotenoproteins, are critically treated. In this section the molecular aspects of photoreceptor function are discussed in detail. The properties of the C_{40} and C_{20} carotenoids *in vitro*, and their radically different features *in vivo* are presented, showing the important role of membrane-protein environment in the light harvesting pigments of photosynthetic membranes and in rhodopsin and bacteriorhodopsin. A great emphasis is laid on the molecular basis of the blue light effect and the possible role of flavoproteins and related redox reactions.

The chapter on bioenergetics presents a brief but very useful survey of irreversible thermodynamical concepts and their application to the secondary and primary active transports performed through biological membranes and to ATP synthesis coupled to redox reactions, in accordance with the Mitchell theory. This chapter is followed by a description of photosynthetic membranes, with emphasis on the role of pigments, including the electric field-dependent absorption shift of carotenoids and chlorophyll b, an internal probe of membrane potential. This chapter is of course rather abridged.

The last section of the book deals with photosensory transduction chains in different organisms.

There are a lot of open questions regarding the actual mechanisms of stimulus and signal transmission, from the excitation of the receptors to the resulting response. However, there are several quite general features, such as the importance of electrochemical gradient across the cell membrane and the possible role of Ca^{2+} ions in motor responses. For this reason, the last chapter is a very useful summary of the sensory transduction chains (including the still hypothetical steps) for several organisms, and it provides some general conclusions.

This volume of the NATO ASI series, as noted in the preface, gives an interdisciplinary approach to photoreception and sensory transduction in aneural organisms. It is a critical overview of the problems and a useful advanced treatise for researchers entering the field.

L. ZIMÁNYI

Comprehensive Virology. Vol. 14., Newly Characterized Vertebrate Viruses (Edited by H. Fraenkel-Conrat and R. R. Wagner) Plenum Press, New York and London, 1977, p. 544.

Volume 14 contains latest results on eight new virus groups of vertebrates. Development of the methodology of molecular biology allowed the establishment of several virus groups comprising previously unclassified viruses or viruses erroneously grouped together with superficially related viruses. Isolation, structure of the virion, replication, genetics and pathogenesis of these newly characterized viruses are discussed with emphasis shifting according to the available information. *Bunyaviridae* (by D. H. L. Bishop and R. E. Shope). Almost hundres viruses formerly classified as arboviruses are now known to meet the criteria of belonging to the family *Bunyaviridae:* the enveloped virions (100 nm in diameter) contain three single-stranded RNA pieces of negative polarity. Two virus-specific glycoproteins are located at the surface of the virions that are formed by budding into the Golgi cisternae. From the diverse nature of the vertebrate host and vectors it is not clear, however, which one is the primary host. *Arenaviruses* (by W. E. Rawls and Wai-Choi-Leung). Far less is known about the molecular biology of arenaviruses. The virion has two single-stranded negative-sense RNA species and, most unusually, it contains ribosome particles as well, whose function is totally unclear. With the exception of Lassa fever virus all other members are maintained in nature by persistently infected rodents in South America. *Coronaviridae* (by J. A. Robb and C. V. Bond). Coronaviruses have been isolated from almost all species of domestic and laboratory animals, and from man as well. Enteritis is one of the main manifestations of the disease caused by them. They are enveloped viruses with one single-stranded RNA species of positive polarity with molecular weight of 6—8 million dalton thus establishing this polynucleotide the longest known mRNA. *Caliciviruses* (by F. L. Schaffer). Some members of picornaviruses have recently been removed from the family Picornaviridae on the basis of their different genome strategy from that of picornaviruses. Caliciviruses, unlike picornaviruses, replicate through a subgenomic mRNA. *Orbiviruses*

(by D. W. Verwoerd, H. Huismans and B. J. Erasmus). Most of these viruses can only be isolated from insects in Africa although diseases caused by them (e.g. blue-tongue) have been known since the turn of the century. By morphology they resemble rheoviruses but some unique properties (smaller size, absence of the second capsid layer, sensitivity to lipid solvents, requirement of an insect vector in transmission) clearly place them into a separate genus. *Icosahedral Cytoplasmic Deoxyriboviruses* (by R. Goorha and A. Granoff). These viruses are widely distributed in nature with hosts including plants, insects, fish and mammals. Members have icosahedral symmetry, replicate in the cytoplasm and contain DNA. *Fish viruses and viral infections* (by P. E. McAllister). Now some two dozens of diseases are recognized in fish the causative agents of which can be classified into at least six established taxonomic groupings. *Viruses of human hepatitis A and B* (by W. S. Robinson). Viral hepatitis is probably one of the earliest recognized infectious diseases although its causative agents were characterized only a couple of years ago. Under natural condition both infect only man. Hepatitis B virus is a small DNA virus unrelated to any other known virus, while hepatitis A fits into the picornavirus group. As a result of a unique form of infection by hepatitis B virus, various structures are found in the blood of patients, all carrying surface antigens (formerly known as Australia antigen).

B. Lomniczi

Biochemistry by P. Elődi, Akadémiai Kiadó 1980, 833 pages, 318 figures.

My opinion may be a very unique one but I believe that biochemists are generally reluctant to carry out experiments in absolute terms, i.e. they relate their observations, results and opinions to something, generally referred to as "control", "normal" or "blank" values. The same holds true for the appraisal of scientific literature. A work, be it either some pages long (or a few sentences in the case of a lecture) or a monography in one or more volumes full of basic statements and principles, invariably needs a "control" when it comes to rating. For the work of Dr. X, the work of Dr. Y (similar in character in dimensions, subject, etc.) will serve as a basis of comparison. P. Elődi's "Biochemistry" cannot be an exception to this. However, in this particular case Dr. X and Y are some of the classics in biochemistry.

It is not by chance, I suppose, that the number of comprehensive textbooks of biochemistry has recently been reduced. Students of biochemistry in the fifties were quite pampered since the two volumes of Phosphorus Metabolism edited by Mc Elroy and Glass in 1952 (based on the material presented at a symposium held in 1951), contained such a wealth of new information that it even sufficed for an entrance exam to obtain a Ph. D. degree. In 1955, the comprehensive, up-to-date bible of the enzymologists, the four-volume Methods in Enzymology by Colowick and Kaplan, was published. In 1958 the first edition of Straub's Biochemistry appeared followed, to the best of my knowledge, by seven more impressions.

Nowadays not too many comprehensive biochemistry textbooks, at least as persisting as the aforementioned Straub's Biochemistry, are being published. In 1967 the book "Concepts in Biochemistry" by Reithel offered something new from a didactic point of view. Instead of providing an immense mass of information, it dealt with some particularly important problems greatly helping thereby the orientation of students and non-biochemists in the information jugle of biochemistry. To illustrate the accumulation of biochemical knowledge, a single example may be indicative: the number of pages of the J. Biol. Chem. was 1120 (in small format) in 1957, while 20 years later, in 1977, it reached 9118 pages in its present large format. And The Journal of Biolobical Chemistry is only one of the leading biochemical journals.

It was as late as 1970 when the library of biochemistry was extended by a new textbook of remarkable qualities, Lehninger's textbook gave on 833 pages such a comprehensive picture of contemporary biochemical knowledge that it helped to a large extent not only the student but also the teacher. It is not by chance, therefore, that in 1972 the 6th impression of this book came to light. Lubert Stryer, in 1975, broke the traditions with his biochemistry textbook in the sense that he gave lesser weight to chemical back-

ground but stressed instead biological relation, aided by extremely well-conceived figures. This textbook also made a very high score.

The textbook of P. Elődi, published in 1980, on almost one thousand pages, must stand comparison with the above-mentioned books. I must say that this reputable work is apt to this comparison in every respect. The author's three-decade long research experience, his attraction to, and talent for teaching resulted in a work in which the fundamental concepts of physics and chemistry are in a healthy balance with the increasingly difficult biological principles. I am personally very much impressed by the rich array of illustrations which very often all by themselves provide enough information which, even for non-biochemists, makes biochemistry an easily understandable subject.

The series of Colowick and Kaplan, originally planned in 1955, to consist of four volumes has recently arrived to the 65th(!) volume. When P. Elődi set out to write his Biochemistry he had to sublime the essence of biochemistry from such an immense bulk of information. The author accomplished this project with admirable virtuosity. What is especially worthy of praise is that there is no bias in the structure of the book in favour of the author's actual research interest in a particular field of biochemistry. He does not emphasize it more than the other parts. Virtuosity refers also to the style. The book is really entertaining, the thoroughly formulated concepts of this excellent author are a pleasure to read.

Nowadays, books with such dimensions and such an enormous body of information are seldom written by a single author. It is especially worth noting in this context that, although delayed by the technical limitations of the Hungarian printing facilities, the attentive reader may easily find data that are a mere few months old.

It is an important question who this book has been written to? I believe that it contains far more information than what is usually required by a college student. It is apparent that with appropriate selection the book can serve as an excellent aid for a student engaged in any field of chemistry or biology. However, the function of the book is much more manifold than that. In my opinion the book offers excellent guidance to all those who, like myself, had been educated on Straub's Biochemistry and for one reason or another had no opportunity to keep up with the recent trends of biochemistry. The book may be equally helpful to all researchers working in the field of genetics, medical, agricultural and other sciences.

I sincerely hope that the book of P. Elődi will soon be published both in English and Russian.

If I were to conclude my thoughts with commonplaces I would say that this book represents a milestone in the Hungarian (and international) literature of biochemistry. The truth, however, is that milestones are readily passed by and this book must not be by passed.

T. Dévényi

Bacterial Toxins and Cell Membranes, by J. Jeljasziewicz and T. Wadström (Eds) Academic Press, London—New York—San Francisco, 1978. p. 432.

Our rapidly growing knowledge of the mode of action of various biologically active substances, such as hormones, drugs, toxins and other compounds, can mainly be attributed — among others — to the up-to-date, suitable methods that nowadays enable us to study these mechanisms not only in the whole cell, but right at their site of action. It shortly turned out that the site of action of a number of biological substances is at the plasma membrane, or more precisely, although these problems are not definitely clear yet, at some kind of intracellular membrane. Studies of the interaction of bacterial toxins and biological membranes not only facilitated the clarification of the mechanism of action of these compounds, but also showed that individual purified bacterial phospholipases, neuraminidases are useful tools in investigations concerning the structure and function of the membrane. So, e.g., cholera toxin proved to be an effective tool for the study of cellular functions regulated by cyclic AMP. Although most of the toxins have been discovered at the beginning of this century, it was only during the past decade that elaborate and intensive research was undertaken.

The present volume provides an excellent survey of this development and indicates many aspects of the perspectives in bacterial toxin research. All the eleven chapters of the volume clerly illustrate the endeavour to integrate at an appropriate level the accumulated knowledge in the field of toxin and membrane research, with fruitful results for both lines.

After an introductory overview Wadström summarizes the progress made in the purification of a number of toxins of protein character. He points out that, in fact, it is only by the use of immunosorbent and affinity chromatography techniques that really complete purification of bacterial protein toxins can be achieved. It follows that most of the literature data — especially the earlier ones — do not contain but results of investigations carried out on heterogeneous and contaminated toxins, and that is why these data cannot be evaluated without due reservations. In the chapter on "Bacterial Endotoxins and Cell Membranes" an excellent attempt has been made to summarize the presently available infoımations on the mode of actions of endotoxins on cell membranes of various types. Despite many years of intensive study the exact means by which endotoxins induce their toxic effects remain to be clarified. It may be that endotoxins in themselves are not "toxic" but the "overstimulation" of otherwise normal processes results in a toxic effect on the host. McCartney and Arbuthnott review the current status of the mode of action of membrane-damaging toxins produced by Staphylococci. They deal mainly with the four principal haemolytic toxins and staphylococcal leucocidin. Smyth and Duncan recapitulate the areas of agreement, discrepancy and ignorance concerning the mechanism of action of thiol-activated cytolysin on biological membranes, they likewise review the production, purification and characterization of the cytolysins. Jeljasziewicz and his colleagues deal with the cytotoxic extracellular products of Staphylococci and group A Streptococci (haemolysins and leucocidins, streptolysins and erythrogenic toxins, resp.). They point out that the study of interaction of several biologically active substances produced by the same microorganisms, supplemented by a much better knowledge of host reactions of these products, should bring a better recognition of their roles in the pathogenesis of streptococcal and staphylococcal infections.

In a very stimulating chapter Gemmel discusses the interaction of bacterial toxic agents with components of the inner mitochondrial membrane involving the electron transfer system as well as the oxydative phosphorylation processes, although the question still exists whether the products of microorganism are able at all to enter the mammalian cell either under in vivo or in vitro conditions. Nevertheless, this problem does not influence the fact that several of these toxins were most suitable tools in the armoury of chemical agents that have been so successful in elucidating the functional organization of the mitochondria. An important question, the streptolysin S-formation and the antitumour activities of group A Streptococci, is treated by Okamato et al. In an outstanding report Gill considers seven toxic peptides that cross the cell membranes. Four of them (Diphteria toxin, Pseudomonas aeruginosa exotoxin A, Cholera toxin and heat-labile enterotoxin from Escherichia coli) are bacterial toxins, Colicin E_3, abrin and ricin are also included since they present the same problems as the bacterial toxins mentioned above. The chapter concentrates on the evidence that those toxins do in fact enter the cells, on their mode of action within the cells and, finally, on observations concerning their interactions with membranes. The experimental basis of our current knowledge about the cholera toxin molecule, its membrane receptor, the structure — function relationship of the toxin and its effects on adenylate cyclase of plasma membrane is condensed by Holmgreen. He also points out where evidence is still incomplete or lacking by evaluating the extent of speculations on the mode of action of cholera toxin without experimental supports. In the last chapter Möllby presents a critical review of the bacterial phospholipases and their interactions with mammalian cell membranes. It is clearly shown from the bacterial as well as the membranologist's point of view what has been possible and what might be possible to learn by the use of phospholipases as probes of structure and function relationship of cellular membrane.

This volume should interest microbiologists, cell biologists, biochemists and all other scientists who are engaged in the research of

membrane structure and function or the effects of bacterial toxins at the membrane level. All these will find interesting, stimulating and fashionable reviews not only of our present knowledge but also on future aspects.

J. Somogyi

Ecological Genetics: The Interface by P. F. Brussard (Editor) Springer Verlag, New York, Heidelberg, Berlin (1978) p. 257.

This book contains the proceedings of the symposium on "Genetics and Ecology: The Interface" sponsored by the Society for the Study of Evolution and held at Ithaca, New York, June 12—15, 1977, with a limited number of outstanding contributors. The aim of this symposium, and consequently of this volume too, was to promote integration of the two rapidly developing disciplines of supra-individual biology: population genetics and population ecology.

One dozen papers are arranged under six chapters. The first one, "Theory" consists of contributions by S. A. Levin ("On the Evolution of Ecological Parameters") and J. Roughgarden ("Coadaptation of Equilibrium Population Size"). Levin's excellent review is both impressive and disappointing. The latter applies to the conclusion: Evolutionary models seem to be explanatory rather than predictive, what is more, "evolution is something which simply happens, rather than a calculated, far-seeing program for optimization." (page 22). Roughgarden analyzes the relationship of strategies that maximize fitness with strategies that maximize population size. This subject unifies the treatments of evolutionary and ecological theories in a co-evolutionary model.

The second chapter is devoted to "Physiology, Biochemistry and Adaptation" with two papers. R. K. Koen deals with an enzyme (leucine aminopeptidase) polymorphism of a marine bivalve in connection with adaptation, emphasizes the need for coordination of physiological and genetic studies. W. J. Schull, R. E. Ferrel and F. Rothhammer report a detailed study of evolutionary adaptation to the hypoxia of higher altitude by the aboriginal Americans.

Chapters 3 and 4 are devoted to research reports on ecological genetics of animals. Three papers (Chapter 3) study natural populations of Drosophila species while under "Other Animals" (Chapter 4) field crickets and snails are investigated in two papers. This 3 : 2 proportion reflects the necessity of both genetic and ecological knowledge of the species studied.

Chapter 5 is particularly important, for relatively few papers are published in the literature on the "genetic demography" of plants. However, the extension of enzyme-polymorphism studies to higher plants revealed some remarkable advantage of the sessile plants over most animals. Age specific selection can often be analyzed directly in natural populations. This is demonstrated by M. T. Clegy, A. L. Kahler and R. W. Allard ("Genetic Demography of Plant Populations"). The same topics is dealt with in more general terms by D. A. Levin ("Some Genetic Consequences of Being a Plant"), a paper which will probably inspire more gene-ecological studies with plant populations.

The last Chapter with a single paper by R. R. Sokal studies population differentiation posing the problem by the dilemma: "Something New or More of the Same". A tentative answer is given in the Hegelian conclusion "more of the same leading to something new".

A similar statement is true of the whole volume. Something new has began to emerge, between the fertile fields of genetics and ecology with the powerful help of mathematical modelling.

G. Vida

Mathematical Population Genetics (Biomathematics Vol. 9.) by Evens, W. J. Springer Verlag, Berlin, Heidelberg, New York (1979) p. 325.

Population genetics has long been a vigorous interface of biology and mathematics. This is partly due to the early activity of the "Great Three": R. A. Fisher, J. B. S. Haldane and S. Wright, who laid down the theoretical basis of population genetics during the 1920s and 1930s. Now, population genetics is a very active field of biology again, since the development of modern biochem-

ical technics (particularly that of electrophoresis and electrofocusing) made it possible to study the state and dynamics of gene frequencies in natural populations, thus matching theories with observations.

W. J. Evens' book is written in a clear, graceful style, understandable to both the mathematically trained biologist and the biologically oriented mathematician. Although an advanced level of mathematical knowledge is obviously necessary to fully understand the logic and kind of reasonings, the formalism is always accompanied by verbal conclusions, thus the book is also useful to the mathematically less trained average biologist (provided they trust the author's treatise).

The book consists of ten chapters. After a very enjoyable historical introduction (1. "The Golden Age"), some technical discussions and rather straightforward generalizations (2. "Technicalities and Generalizations"), an up to date single locus treatment is given on the "Discrete Stochastic Models" (Chapter 3) and the "Diffusion Theory" (Chapter 4) with its applications (5. Applications of Diffusion Theory). Evens then steps further, introducing some two loci models (Chapter 6) with discussion on the evolutionary importance of recombination, and extending the problem to many loci (Chapter 7). Here the reader will agree with Evens' comments in the introduction: "The complexities of the genetic behaviour of populations, as shown by the (still incomplete) mathematical theory, are far greater than our power to comprehend and control." The next chapter (8. "Molecular Population Genetics") is probably one of the most exciting topics of modern biology. Here we can see the evolutionary process in a retrospective manner, dealing with "real entities" such as the four kinds of nucleotides in the chain of DNA. The ninth chapter ("The Neutral Theory") deals with the most controversial question of evolutionary genetics. The large amount of genetic polymorphism recently revealed in natural populations of almost every sexually reproducing organism is not examplified here but rather the mathematical arguments leading to the neutral theory and their deductive properties are presented. Also, some mathematical procedures are given to test the neutral hypothesis. The last chapter is devoted to "Generalizations and Conclusions".

The quality of printing is very good, which is particularly important in a book full of formulas and tables. A few misprints, unfortunately, seem to be unavoidable (e.g. scheme 1.91).

This book is warmly recommended to everybody interested in theoretical population genetics and evolution. Although the book is not attempted to cover the whole field of population genetics, it is certainly a most inviting presentation and probably the best summary of the state of art in neutral mutation hypothesis and treatments of two and more loci.

G. Vida

Mikrochemische Methoden für neurobiologische Untersuchungen (Microchemical Methods for Neurobiological Research) by Peter Uwe Witte and Heinrich Matthaei. Springer Verlag, Berlin, Heidelberg, New York, 1980.

The book contains a selection of the most important micromethods in the field of synaptical signal transformation: 38 methods- for enzyme activity, transmitter and metabolite determination that were tested and further developed by the authors and 17 others taken from the literature. In the first part procedures of tissue preparations for the different assays, tissue and cell cultures are dealt with. Histological staining, histochemical, and fluorescent methods are included, also examples of calculations of enzyme activities using labelled substrates are given and the source of the equipments necessary for the experiments are mentioned. On the 141 pages of the book the most important methods of neurobiology are practically and clearly summarized.

Maria Wolleman

Female Sex Steroids. Receptors and Function • Monographs on Endocrinology. Vol. 14. by Clark, J. H. and Peck, E. J. Jr. Springer Verlag, Berlin, Heidelberg, New York, 1979, p. 245.

Knowledge of the steroid–receptor interactions and the hormone-induced response of target organs progressed considerably in the

last decade. Research on the steroid hormone receptor function provided additional insight into the molecular mechanisms responsible for the maintenance of tissue-specific effects of steroid hormones.

The authors' work fuses our knowledge of the presence and behaviour of tissue-receptors of female sex steroids and their function under normal and pathological conditions.

In the introductory chapter of the monograph, methods of steroid receptor characterization and the determination of receptor parameters (saturation, binding systems, specificity, receptor assay) are summed up. A series of chapters give the common basis of cytoplasmic and nuclear receptor forms, features of nuclear binding and biological response following translocation of cytoplasmic receptor–steroid complex, and the control of steroid receptor levels under physiological conditions.

A fascinating section of the book is the description of steroid antagonisms at the cellular level, the interaction of various steroids to change receptor concentrations and the pharmacological alteration of the sex steroid hormone receptor contents in target organs.

Research on the hormone dependence of certain malignancies and the receptor content of cancerous tissues is a promising tool for prognostic and therapeutic evaluation of certain forms of cancer in females. This excellent volume gives an up-to-date and thorough summary of our recent knowledge of this important field.

Gonadal steroids play a decisive role in the endocrine function of the central nervous system. A chapter of the monograph describes the newly discovered interactions between gonadal steroids on one hand, and sexual differentiation during prenatal life, female reproductive functions or age- and sex-related differences of the brain function, on the other. An outline of the molecular mechanisms at this level is one of the most valuable section of the book.

Finally, the author's speculations and conclusions are summed up on the basis of both the comprehensive literature and the fundamental work of their laboratory.

T. Fehér

The Geometry of Population Genetics by E. Akin (Lecture Notes in Biomathematics, Volume 31) Springer Verlag, Berlin, Heidelberg, New York (1979) p. 205.

Those who are familiar with textbook models of population genetics and evolution will probably be able to digest most of the titles of this remarkable book. An average student of biology, however, I am afraid, will not profit very much from this volume. The text is fairly difficult, assuming advanced knowledge of mathematics (linear algebra and differential equations). Consequently Akin's book can be recommended only to those teachers and research workers (very rare, indeed), who are well trained in both mathematics and populations genetics.

Ever since J. B. S. Haldane, R. A. Fisher and S. Wright described selection by the well known linear equations several attempts have been made to solve or study them. Fairly sophisticated solutions were given earlier by Haldane and Jayakar (Journal Genet. *58*, 291–317). Akin studies these non-linear differential equations in a new way using Shahshahani's method of differential geometry. This approach has proved to be fruitful in the clarification of Fisher's "Fundamental Theorem of Natural Selection" as well as Kimura's "Maximum Principle" to mention but a few.

Among the applications, a very interesting and unexpected cycling has been found in the two-locus-two-allele model of selection plus recombination (with constant fitnesses).

The above examples illustrate the biological relevance of differential geometric analysis which, unfortunately, will not be accessible in this form to "the biologist" readership. A more readable presentation (such as Evens' recent book on Mathematical Population Genetics, Springer Verlag, 1979) would have helped to enter this approach in population genetics on a broader basis.

G. Vida

Pharmacology of Ganglionic Transmission. Handbook of Experimental Pharmacology. Vol. 53. by D. A. Kharkevich (ed.) Springer Verlag, Berlin, Heidelberg, New York. p. 531.

This new volume in the series of the Handbook of Experimental Pharmacology, edited by Prof. D. A. Kharkevich (Moscow) is de-

voted to ganglion blocking agents. By recruiting an excellent international team of contributors, Kharkevich did a superb editorial job with remarkable modesty (he did not contribute himself any chapter except for the Introduction).

In the first chapter, V. I. Skok (Kiev) summarizes present ideas on the morphology and physiology of ganglionic transmission. In contrast to the generally held view, the very first diagram of the book shows, in addition to preganglionic axons, also terminals of afferent fibers which seem to establish synapses upon autonomic ganglion cells. Such local reflex arcs were, in fact, described by members of the Kiev school; there is, however, no general agreement about the existence of such intraganglionic reflex arcs. No less difficult is it to understand why the author did not try to select a better electron micrograph to demonstrate ganglionic synapses; hundreds of good quality pictures are available in the pertinent morphological literature.

The second chapter, by Klingman, Organisciak and Klingman (Buffalo and Dayton, resp.) is a brief but highly instructive summary of ganglionic metabolism. Methods for the examination of ganglion-blocking activity are discussed *in extenso* by L. Gyermek (Davis, California). In this excellent review (Chapter 3), *in vivo* and *in situ* preparation (superior cervical ganglion of the cat, stellate, inferior mesenteric, celiac, ciliary and salivatory ganglia, etc.) as well as isolated organ and isolated ganglia preparations are described and critically evaluated. This Chapter is undoubtedly one of the most useful parts of the whole volume. V. Trcka (Prague) discusses the relationships between chemical structure and ganglion-blocking activity of quaternary ammonium compounds (Chapter 4a) and those of tertiary and secondary amines (Chapter 4b). This is followed by an excellent analysis of the site and mechanism of action of ganglion-blocking agents (Chapter 5) by D. A. Brown (London). D. M. Aviado (Morristown, N. J.) wrote two contributions: Chapter 6, on the action of ganglion-blocking agents on the cardiovascular system, and Chapter 13, on the ganglionic activity of cardiovascular drugs. Both topics are of major practical importance. Recently, in some countries, ganglion-blocking drugs have been (prematurely) displaced by adrenergic neurone blockers and β-blockers; according to Aviado there is good reason to suggest continued therapeutical use of the good old ganglion-blocking agents. On the other hand, interactions between ganglion-blocking drugs and cardiovascular drugs, including antihypertensives, vasocontrictors and cardiotopics, like reserpine, guanethidine, norepinephrine, angiotensine and digitalis, represent potential hazards in therapeutics. Unfortunately, this latter Chapter seems to be less elaborated than deserved. — Also D. F. J. Mason (London) contributed two essays; Chapter 7, on the action of ganglion-blocking agents on the gastrointestinal tract and Chapter 8, on the absorption, distribution, fate and excretion of ganglion-blocking compounds. A major review by R. L. Volle (Farmington, Connecticut) is devoted to nicotinic ganglion stimulating agents (Chapter 9). In this context, the bi-phasic sequence of depolarization followed by hyperpolarization of the ganglion cells, and the elimination of hyperpolarization *via* suppression of the electrogenic sodium-pump mechanism, are discussed in detail. In another contribution (Chapter 12) Volle summarizes the ganglionic actions of anticholinesterase agents, as well as those of catecholamines, neuromuscular blocking agents, and local anesthetics. In this paper, *inter alia*, G. B. Koelle's classical discoveries regarding the dual localization of acetylcholinesterase in autonomic ganglia, and the dual role of acetylcholine in ganglionic transmission, are discussed in the light of recent physiological and pharmacological investigations. Non-nicotinic chemical stimulation of autonomic ganglia is the topic of an outstanding chapter (Chapter 10) by W. E. Haefely (Basel). The actions of acetylcholin, muscarine, metacholine, pilocarpine, carbachol, arecholine, choline, oxotremorine, furthretonium and cholinesterase inhibitors are discussed in terms of the theory on a possible involvement of a second messenger (cGMP) in muscarinic excitation of autonomic ganglia. Effects of other stimulants (5-hydroxy-tryptamine and related indolealkylamines, histamine, angiotensin, bradykinin and cardiac glycosides) are also registered in due order. Haefely concludes that while all autonomic ganglion cells are synaptically excited by acetylcholine through the interaction with nicotinic acetylcholine receptors,

certain cells may be stimulated also through muscarinic receptors. Sensitivity to muscarinic stimulation varies to a great extent. Autonomic ganglia can be stimulated also by circulating endogenous mediators, as well as by cardiac glycosides and veratrum alkaloids; chemical stimulation by potassium is, however, hardly specific. — In Chapter 11, A. Nisti and J. P. Quilliam (London) discuss ganglionic activity of centrally acting neurotropic agents, like volatile anesthetics, CNS depressants, neuroleptics, antidepressants and anti-mania drugs. Therapeutical application of ganglion-blocking agents in internal medicine is treated by E. V. Erina (Moscow) in Chapter 14, while in the last paper of the book (Chapter 15, by A. A. Bunatian and A. V. Mescherjakov, Moscow), the role of ganglion-blocking agents in anesthesiology is discussed with indications, contraindications and other clinical remarks included.

On the whole, Pharmacology of Ganglionic Transmission is a very useful reference book, dealing with many aspects of this vitally important question. Scientists working in various fields related to impulse transmission, be it physiology, pharmacology or experimental morphology, will hardly do without the immense amount of information carried by this volume.

B. Csillik

Mathematical Modelling and Computers in Endocrinology by McIntosh, J. E. A., McIntosh, R. P. (in: Monographs on Endocrinology, Vol. 16. eds: F. Gross et al.) Springer Verlag, Berlin, Heidelberg, New York, 1980, 337 pages, 73 figures, 57 tables, 125 equations, 292 refs.

To many endocrinologists mathematical modelling seems to be synonymous with so-called "whole-system" modelling. In such investigations mathematical methods have been used to simulate quantitatively whole system behaviour by combining data obtained from separate subsystems.

However, the modelling of whole systems is but one aspect of a much more fundamental activity required for any quantitative (and many qualitative) investigations in endocrinology, or science in general. It is this excellent book which provides the reader with the "know-how" of mathematical and computational tools successfully applied in quantitating hypotheses on the biological control of endocrine systems. One of the most attractive features of the monography is that it turns mathematical principles into biological ones and vice versa. I am sure that both mathematicians and endocrinologists will profit from the valuable experience of the authors.

The scope of the book is as follows: §1 discusses in general terms the purposes, advantages and limitations of mathematical modelling, and some of the mathematical and biological concepts involved. §2 is concerned with the characteristics of mathematical techniques appropriate to the formulation of a varied range of biological models; these are presented in general terms. §3 shows how models can be compared with data, and illustrates how to determine when a model is adequate, or requires modifying. §4 describes statistical techniques for designing effective experiments. §5 and §6 deal with several subjects of endocrinological interest to which the quantitative methods of earlier chapters are applied. §7 is concerned with rhythmic processes. The first part describes how theories of cyclic processes have begun to be applied in modelling biological rhythms, while the second outlines methods for analysing rhythmic behaviour empirically. §8 illustrates mathematical modelling of the ovulatory cycle, a large endocrinological system.

In contrast to most of the models described elsewhere in this book, §9 presents model of random processes in endocrinological research. As to the evaluation of these models, two approaches are illustrated: non-parametric statistical methods: Wilcoxon, Mann-Whitney and critical range tests, Friedman and Kruskal-Wallis analyses; parametric multivariate analysis: principal components, factor-, cluster- and discriminant analyses. In §10 guidance is given to statistical formulae and significance tests (parametric and non-parametric, respectively) referred to in the text.

The programs and subprograms (listed and described in §11) will hopefully give inspiration for getting started on modelling the results of analytical experiments. Three main programs of similar structure (together with their common subprograms) are provided: i) fitting equations of models to experimental

data; ii) model-evaluation and Monte-Carlo simulations; iii) designing sequential experiments to discriminate between rival models and estimate parameters efficiently.

From §12 the reader can learn analytical integration of differential equations of compartmental models.

Referenced books, reviews and papers (until 1978) are all carefully selected; they are even fundamental in preparing the reader to apply mathematical modelling and computers in endocrinology or related fields of biology.

F. BARTHA

Gene Regulation by Steroid Hormones by A. K. Roy and J. H. Clark (Eds) Springer Verlag, New York, Heidelberg, Berlin, 1980.

In the last two decades endocrinological research turned definitely to biochemistry. In consequence, a new discipline called "molecular endocrinology" has developed. How gene expression is regulated in eukaryotic cells is one of the most interesting problems in molecular biology. Investigation of steroid hormone action is a very good approach to try to solve this problem.

This book is an offshoot of the Conference on Molecular Mechanism of Steroid Hormone Action held at the Oakland University (USA) in the fall of 1978. Thus it represents a current and up-to-date view of the molecular biology of hormone action. The volume deals with the mechanism of action of almost every steroid hormone: glucocorticoids, sex steroids and the insect steroid hormone, ecdysone. Unfortunately, there are no papers included about mineralocorticoid action. The regulation of gene expression is discussed by taking a wide range of normal and malignant cells: those of the uterus, oviduct, liver, mammary gland as well as various cell cultures (HTC, fibroblasts, pituitary cells).

A very intersting and new field in steroid hormone action is the mechanism of action of the insect molting hormone, ecdysone. Using a number of Drosophila mutants and cell lines the authors can give a recent model of Drosophila gene expression.

Perhaps the best studied system in steroid hormone action is the chick oviduct where the structure and regulation of two genes (ovalbumin and ovomucoid) have been extensively studied. In *Gene Regulation by Steroid Hormones* new data are compiled about this system, too.

Following the papers critical discussions by experts in this field are given, stimulating ideas and experiments to clarify points in steroid hormone action.

This book certainly will do service not only to investigations of steroid hormone action but also to molecular biologists as well as medical students interested in this field.

ANIKÓ NÁRAY
I. HORVÁTH

Photosynthesis II. Photosynthetic Carbon Metabolism and Related Processes by M. Gibbs and E. Latzko (eds) Volume 8. Encyclopedia of Plant Physiology, New Series, Springer Verlag, Berlin, 1979. p. 587

It is uncommon that a new edition is thinner than the former, but this is the case with this new comprehensive review of the carbon pathways in photosynthesis in volume 6 of the Encyclopedia of Plant Physiology, New Series. The concise nature is probably due to an integration of the previously established facts with those more recent aspects of research in which substantial progress has been made. The overall coverage of the book remains complete and the fifty-one contributors provide an unusually lucid picture of the current status of research. Under the heading "The reductive pentose phosphate cycle" (five chapters) regulatory processes are outlined and special attention is given to the methodological problems of isolating and reconstituting chloroplast systems. The chapter devoted to prokaryotes is an interesting demonstration of the diversity of the Calvin cycle, due to the low degree of subcellular compartmentation in these organisms. Under the topic "The C_4 and Crassulacean Acid Metabolism Pathways" (five chapters) the reader finds a critical analysis of the experimental facts and open questions concerning the regulation and energetics of these specific but important pathways of carbon metabolism. Short but particularly interesting chapters describe both the rhythms of enzyme capacity and activity as adaptive mechanisms

and the implications of carbon isotope fractionation by plants. The title "Factors influencing CO_2 assimilation" headlines five chapters most of which are very condensed surveys of the facts which provide a few explanations. "Biochemical Basis of Ecological Adaptations" is a section of particular importance. It highlights one of the rapidly developing areas in photosynthesis research. The next seven chapters cover the "Regulation and Properties of Enzymes of Photosynthetic Carbon Metabolism". This part illustrates very successfully the relationship between photosynthesis and the other processes of carbon metabolism and demonstrates by interspecies variation, the diversity of photosynthesis. In the nine chapters grouped under the heading "Ferredoxin-Linked Reactions" the relationship between photosynthetic carbon and nitrogen metabolism receives an individual treatment. The chapters detailing photosynthetic assimilation of sulfur compounds and hydrogen metabolism are particularly elegant and succinct reviews.

To sum up, this volume once again has achieved its avowed purpose of providing timely reviews on the basic and expanding areas of investigations in photosynthetic carbon metabolism. One of its greatest values may lie in pointing out how much remains to be done.

Ágnes D. Faludi

Pathophysiology, by Buehlmann, A. A. and Froesch, E. R. (Eds) with the contributions of Baumgartner, G., Frick, P. G., Knoblauch, M., Lichtlen, P., Scheitlin, W. A., Schmid, M., and Straub P. W. Translated from German by Tegler, T. Springer Verlag, New York—Heidelberg—Berlin, 1979, 403 pages, 74 Figures.

This is already the fourth monography published recently that may be specified as belonging to the "patho" series. It is unambiguous that these volumes reflect certain traditions, as all of them originate from German-speaking areas. The question arises whether or not pathophysiology or pathobiochemistry can be considered as an internationally acknowledged, relatively independent branch of science. If we think it over more thoroughly the answer is principally negative. Still, for practical reasons, publishing of such monographs seems to be instified. The help the studies of medical students and also may prove to be important to clinical physicians, who work in different fields and intend to understand the physiological or biochemical basis of a given process or phenomenon or want to refresh their memories about these matters.

This concisely compiled volume is a translation of the third German edition. Each chapter begins, logically enough, with a brief description of the mechanism of the normal processes followed by an explanation of the origin and consequences of malfunctioning.

The structure and content of the chapters remind the reader in many respects, of those of the textbooks of internal medicine. Actually, certain chapters would better suit the texture of the latter since there they would help the practitioner to *understand* why the symptomps of his patient have developed the way he observes them.

The book discusses in 12 chapters arranged essentially according to individual organs, the physiological problems underlying a pathological events. One chapter has usually been written by one or two of the contributors.

The book starts with a description of the lungs and respiration followed by a discussion of the problems of the heart and circulation. A short part of not more than six pages deals with thermoregulation. In Chapter 8 blood is reviewed. This may provide a good example to illustrate the impossibility to cover, on a mere 40 pages, except some definitions and summarizing tables, all the problems of the particulate elements of the blood, the leukemias, anaemias, the immune system, blood clotting, the plasma proteins and porfiriaes. The text and the tables are satisfactory as they provide enough information to the reader where to get more information about the subject of his particular interest. It would have been very useful, however, if a list of the recommended literature had been included at the end of the individual chapters.

Chapters 5—7 discuss the kidney, as well as water-electrolyte and acid-base equlibria. These two latter topics are usually neglected area in both biochemical and physilogical textbooks a fully unjustified practice which

is difficult to explain. The more so, because it is often inevitable for the practitioner to be aware of the underlying principles of water–electrolyte and acid–base equilibria to save liver. A short chapter is about the bone and the calcium and phosphate balance related to it.

Chapter 9 about the hormones provides the reader with a true, classical physiological picture, clearly pointing out that physiology and pathophysiology are not independent, unrelated areas. Usually most physiological works on normal processes also discuss hormones by starting from anomalies of the hormone system. The only thing a biochemist misses somewhat is that, because of the physiology oriented way of thinking of the authors not even a brief survery on the molecular mechanism of hormone action is presented. A molecular approach would have enabled the authors to give an up-to-date interpretation of some very important principles of regulation such as the ying-yang mechanism (regulation *via* two counteracting processes or systems).

Chapter 10 discusses metabolism on a mere 40 pages. Almost half of this limited size is devoted to diabetes mellitus. This is an outstanding theoretical and practical example of how the relationships between and mutual regulation different metabolic pathways can be clarified. Just here the reader would like to find a compilation of the numerous but, fortunately, rather infrequently occurring metabolic disorders. Unfortunately a description of even the most common one, phenylketonuria is missing. This is a regrettable omission since again an excellent chance has been missed to demonstrate the relationship between a given malfunctioning of metabolism and the resulting damage to the nervous system.

The last two, longest chapters describe the digestive tract and the nervous system. The former one has constituted the main subject of classical physiology for decades, thus, it is relatively well known. As for the function of the nervous system, the past decade brought to light a plenty of new and interesting findings. That is why the biochemist feels a need to learn somewhat more about, for example, the neurotransmitters, the numerous stimulatory and inhibitory exogenous substances affecting the nervous system, the effect of many of which is met nowadays by practitioner as well.

Despite the above shortcomings, this volume offers a lot of information, is well compiled and the fundamental principles are well formulated.

P. Elődi

Placental Proteins by A. Klopper and T. Chard (eds) Springer Verlag, Berlin, Heidel berg, New York, 1979.

The book which gives a lot of up-to-date information on the subject mentioned in the title is based on the latest meeting of triennial conferences, held in Aberdeen, 1978. It deals with those new proteins of trophoblastic origin that have been purified recently and can be considered as really pregnancy-specific substances.

The first chapter gives an overview of the specific proteins of human placenta, the second one deals with the measurement of trophoblastic proteins while the third chapter introduce the potential antifertility vaccines using antigens of human chorionic gonadotrophin (hCG).

Two further chapters deal with the isolation of placental proteins as well as their characterization. In the fourth chapter a very brilliant and unusual method, negative antibody affinity chromatography completes the common separation methods (e.g. ion exchange chromatography, gel filtration, etc.) used in purification. The isolation steps are followed by descriptions of the adequate analytical procedures. The fifth chapter also presents the isolation work, but it consists of a more comprehensive presentation of various origin.

Each of the last six chapters deals in detail with one or two special kinds of pregnancy-specific or pregnancy-associated proteins. Especially, the sixth chapter seems to be a brilliant work on the "Pregnancy-Associated Plasma Proteins: PaPP-A and PaPP-B". This chapter presents the identification, purification, characterization, synthesis, physiology of PaPP as well as the role of PaPP in pathological states. The figures and tables illustrate very well this short but valuable chapter. The seventh part presents not only

the isolation of pregnancy-associated plasma protein-A but also some fine and interesting observations on the isolation process. Nevertheless, I find that the pictures are not satisfying enough with respect to the homogeneity of PaPP-A after the final step of purification. The eight chapter gives a comprehensive study of trophoblast-specific β_1 globulin (SP_1) and placental protein-5 (PP5) in early pregnancy showing the changes in their level in normal pregnant subjects during the gestation period and after delivery. The ninth chapter gives the clinical aspects of pregnancy-specific β_1 glycoprotein (PSβG). The tenth chapter details and scans the possibility of measuring PSβG. The manifold methodlogical considerations further expanded by a pictorial and tabulated presentation of the results and the data obtained. The final chapter gives an excellent review of the recent clinical studies of thromboplast-specific glycoproteins.

The whole book provides a concentrated picture of the topic, the valuable information contained in the text is well illustrated with figures and pictures of extra good quality. Each chapter is completed with a recent reference list in alphabetical order.

This interesting volume can be recommended to everybody who works with or is interested in the isolation, characterization, analysis, biological role and clinical aspects of placental proteins and to researchers who intend to expand their experience about these proteins.

H. KALÁSZ

Neonatal Screening for Inborn Errors of Metabolism by H. Bickel, R. Guthrie, G. Hammersen (Eds) Springer Verlag, Berlin, Heidelberg, New York, 1980.

It is common practice that the full text of the lectures delivered at a symposium dealing with special problems is edited in the form a book. It is also customary that these books arouse limited interest. But I think the volume to be reviewed is an exception.

In September 1978 a remarkable symposium was organized, concerning the neonatal screening for inborn errors of metabolism. The book contains the full, comprehensive text of this symposium.

Since the introduction of Guthrie's method in 1963 for the early detection of phenylketonuria (PKU) 4, one of the most frequent inborn errors, more than 34 million newborns were tested until 1978, and more than 3000 cases of PKU have been diagnosed. The greatest merit of the symposium was that the participants summarized the achievements in this field and showed the future possibilities.

The symposium had three main topics. The material presented clearly shows how these problems appear in every-day practice.

The first part deals with the screening for hereditary metabolic disorders. Since the principles and methods of mass screening as well as the therapy and general care of the patients are well established, the participants briefly summarized the experiences gathered in the last years. The chapters dealing with the common amino acid disorders and galactosemia were exposed by the most prominent experts.

Early detection of organic acidurias in newborns arose as a new problem. These life threatening disorders are rare, but selective, multidimensional screening procedures have to be worked out to achieve effective results.

In addition to aminoacidopathies there are many other inherited diseases with certain metabolic background. Various methods were developed for the early recognition for example of cystic fibrosis, hyperlipidemias, muscle dystrophy and so on, but no reliable method is so far available. Therefore to introduce mass screening for these diseases is highly questionable.

The second part dicusses on more than 100 pages the problems of hypothyroidism screening. We agree with Dussault that congenital hypothyroidism is almost the ideal model for mass screening programs. The frequency of this disorder is at least twice as high as that of PKU. Reliable, highly sensitive and relatively simple screening methods are available and treatment is also settled. From the papers trying to find a suitable method the reader cannot decide whether the TSH or the T_4 test should be preferred, but one thing is clear, screening for hypothyroidism must be introduced. Methodological and organizatory problems are dis-

cussed, based on more than 1 million examinations performed in the last 5 years. Summarizing the various opinions, measurement of TSH on the 5th day of life, by radioimmunoassay gives the most satisfactory results.

The title of the third part "Neonatal screening programs, orginatization, legislation, methodologic pitfalls and quality controls" shows how many paramedical problems have to be solved if a mass screening program for newborn infants is to be launched. In the introductory lecture Guthrie summarized the results of the last decade. He emphasized that one of the greatest achievements of modern preventive medicine was the introduction of large scale biochemical screening of the newborn population in order to prevent the development of mental retardation.

According to the recommendations of the symposium the following diseases should be definitely screened: PKU, galactosemia, hypothyroidism and maple syrup urine disease. For the following disorders screening tests exist, but cannot be recommended for various reasons: tyrosinemia, homocystinuria and histidinemia. Urine screening can for technical reasons not yet be recommended, but if suitable methods become available organic acidurias can be included in the program. Routine screening for cystic fibrosis is not recommended, screening for hemoglobinopathies, lipidemias is preferable among high risk populations, but for muscular dystrophy it is not justifiable at all.

This short review cannot reflect the spirit of the symposium. The writer of these lines, an ardent worker in the field of prevention of mental retardation, appreciates the enormous development which has been achieved in this field.

From the point of view of biochemistry and biochemists there are many important considerations. This book, strictly spoken the symposium, has been addressed first of all to the clinicians. However, in this field multidisciplinary team work is increasingly gaining ground and in this the biochemists have an equal and fundamental shore. The efficiency of well organized mass screening programs is based upon modern biochemical methods. For the biochemists this book is a very exciting reading, since it shows that the whole society is the beneficiary of their basic research. It is also highly recommended to all those who deal with the education of biochemists and geneticists, because throughout the text the practical implications of apparently abstract basic research are clearly shown.

The edition is excellent. A short but important and well selected reference list is added to each chapter. The tables and figures are suggestive and offer great help to understand the text. The typography bears the usual high standard of the Springer Verlag. This remarkable and important book is concluded by a detailed subject. index.

P. Kiss

Magnetic Resonance in Biology. Volume 1. by Jack S. Cohen (ed.), John Wiley and Sons; New York, Chichester, Brisbane, Toronto: 1980.

This book is the first volume of a new series of Wiley-Interscience Publication.

Nuclear Magnetic Resonance (NMR) provides valuable information on a wide range of systems: from small molecules through macromolecules to cells and whole organisms. The aim of this book is to provide a forum for experts of NMR to describe the applications of this method to biological systems in a form that is also comprehensible to non-experts. This volume consists of six more or less independnet reviews on topics of great interest to biologists and biochemists.

In the first chapter "Intramolecular Dynamics of Proteins and Peptides as Monitored-by Nuclear Magnetic Relaxation Measurements" by Robert E. London an effort has been made to summarize the NMR relaxation behaviour resulting from different dynamic models which describe motions inside macromolecules. Conformational changes of enzyme molecules have been proposed to have an important role in catalysis. Correlation of dynamic data derived from NMR can provide a clearer picture on the functionally important motions of these macromolecules.

The second chapter entitled "Medical Imaging by NMR" has been written by David I. Hoult. This review explores an area which provides new possibilities in research

and clinical practice. The author discusses the sensitivity, the speed and the possible future application of this method.

Chapter 3 "NMR Studies of Drug Metabolism and Mechanism of Action" by Gerald Zon presents studies which show that quantitative NMR analysis of drugs and related pharmaceuticals is still an active field of research. This review covers subjects of intense biological research activity in which NMR has made significant contributions to correlate chemical structure with biological mechanism.

The next chapter "Biosynthesis and ^{19}F NMR Characterization of Fluoroamino Acid Containing Proteins" has been written by Brian D. Sykes and Joel H. Weiner. In most applications of NMR for the study of proteins ^{1}H NMR has been used. These authors suggest that the use of ^{19}F as the label and ^{19}F NMR as the method is an alternative approach which provides various advantages. The way to prepare fluorino-labeled proteins and the interpretation of ^{19}F NMR spectra are also discussed.

Chapter five "Structure of the Capsular Polysaccharide Antigens from Haemophilius influenzae and Neisseria meningitidis by NMR Spectroscopy" by William Egan deals with (a) general features of the ^{13}C NMR spectra of sugars and polysaccharides, (b) structural studies of H. influenzae and N. meningitidis type-specific polysaccharides and Escherichia coli capsular polysaccharides, and (c) the microdynamic behaviour of capsular polysaccharides. The purpose of the author is to demonstrate how NMR spectroscopy can be used to investigate correlations between chemical structure and biological function.

In the last chapter on "Nucleic Acid Structure, Conformation and Interaction" by Martin P. Schweizer an overview of results on NMR studies of polynucleotides, RNA-s, DNA-s, nucleosome particles and structural units of genes is given.

The six reviews of this book represent some of the most important applications of NMR methods in biosciences. It will be a valuable resource book for biochemists, physical chemists and molecular biologists and it may promote future NMR research in biological sciences.

I. Simon

Protein Methylation by Woon Ki Paik and Sangduk Kim John Wiley and Sons, New York, Chichester, Brisbane, Toronto, 1980, 282 pages, 25 figures, 34 tables.

This book is the first volume of a series of monographs on biochemistry.

The authors, leading authorities in the field, demonstrate the very distinct but complementary aspects of protein methylation which is one of the most common post-translational modifications of protein amino acid residues in both eukaryotic and procaryotic organisms.

The history of protein methylation is relatively short when compared with other modification reactions. During the intervening years it has been discovered that the phenomenon of protein methylation is far more complex and diverse than originally thought.

This timely book is the first and only comprehensive review of present knowledge on protein methylation. Although the book consists of sixteen independent chapters, care has been taken to harmonize them. In the discussion of the different topics of protein methylation, in addition to historical aspects methodological questions are given special emphasis. These latter include (i) all known methods to separate various methylated amino acids or peptides from non-methylated compounds, (ii) methods for the synthesis of various unusual, newly discovered methylated amino acids; (iii) procedures for the assay and purification of protein methylases. The biological significance of free and bound methylated amino acids (e.g. chemotaxis and protein-carboxyl methylation) and some interesting aspects of free methylated amino acids (e.g. occurrence of methylated amino acids in nature, pharmacological effects of methylated amino acids), are also dealt with.

Most of the proteins in which methylated amino acids have been detected are structural proteins or carriers such as histones, flagellar proteins, encephalitogenic brain protein, cytochrome C or proteins endowed with complex biochemical and biophysical properties such as myosin, actin, or rhodopsin. In the past, N-methylation of bound L-lysine has been most extensively studied, but now it is known that arginine, dicarboxylic amino acids, histidine residues of protein molecule can also be methylated. In addition, bac-

terial ribosomal proteins have recently been found to contain other methylated amino acid derivatives, too. The discovery of the first protein demethylase is a very important step towards a better understanding of the dynamical relationship between protein methylation and demethylation. The formation of formaldehyde by demethylation and the reactions of this aliphatic aldehyde in biological systems suggest that protein methylation and demethylation may be a part of formaldehyde metabolism.

In spite of the important advances in the field of protein methylation, a number of critical questions are still unanswered or only partially resolved by experimentation. These are the biological significance of protein methylation and demethylation, the timing of methylation in relation to the synthesis of the polypeptide chain, the reactions of the formaldehyde formed by demethylation, etc. It seems that the investigation of the biochemical significance of protein methylation (and demethylation) has only just begun.

The book is distinguished by precise formulation as well as clear definitions and explanations of terms. The value of the book is further enchanced by numerous references at the end of each chapter.

This volume will be a useful guide and source of reference for all biologists, molecular biologists, biochemists and biochemistry who are interested in subjects relating to the protein methylation.

E. Tyihák

Kinetics of Fast Enzyme Reactions. Theory and Practice. by K. Hiromi, Kodansha Ltd., Tokyo and J. Wiley and Sons, New York—Chichester—Brisbane—Toronto, 1979. p. 346 + vii.

The book surveys the development of enzyme kinetics, gives a brief historical summary of the steady-state kinetics. The attention of the author is mainly focussed on transient kinetics which offers far more direct and useful information about enzymatic reaction mechanism, including the changes in the enzyme molecule itself.

Transient kinetic studies require special techniques and theoretical treatment. Very few books are available which deal in detail with transient kinetics. Therefore this comprehensive treatment of the theory and practice, including all possible artefacts, is extremely useful. However, it seems a morbid joke of the editor to present the book with an artefact on the frontispiece (identical with Fig. 2.46.b). Or this would be a criticism concerning the experimental results of the possible readers?

After an introduction to enzyme kinetics (history, analysis and steady-state kinetics of enzyme reactions), the book deals with measurement of rates of fast reactions in solution (stopped-flow, continuous-flow, rapid quenching, temperature-jump, pressure-jump, electric field-jump, flash photolysis and pulse radiolysis methods), with the observation of enzyme reactions (optical properties of the enzyme, of ligands, indicators and probes), with the analysis of fast enzyme reactions (data processing for relaxation time evaluation, kinetics of fast reactions in solution, analysis of transient phase), with mechanistic interpretation of kinetic data, and with some useful but far from being comprehensive examples of the analysis of fast enzyme reactions.

The main goal of this book for the experimentalist is the probably complete list of possible artefacts in rapid kinetic techniques, illustrated with abundant and expressive figures.

The theoretical part is good and moderate. It is an excellent guide for the experimentalist to understand the theoretical basis of transient kinetics and to evaluate properly the experimental data. It contains the evaluation of: 1. the first order rate constant of a single exponential curve, 2. reaction curves involving two exponential terms, 3. single-step relaxation, 4. multi-step relaxation, including parallel and consecutive reversible elementary steps, 5. far-from-equilibrium reactions, 6. pre-steady-state (two and three step mechanisms), 7. single turnover, and 8. non steady-state (where the concentration of the enzyme and that of the substrate are commensurable).

The book is an excellent guide for researchers to understand and perform research in fast kinetics of enzymes, but it is probably too specialized for students. Therefore the exercises at the end of each chapter seem to be superfluous. The references are good in-

dicators to be absorbed further in rapid kinetic work in enzymology.

The book may be recommended to all laboratories where fast kinetic techniques are used.

T. KELETI

Some 150 conversion tables make the book a useful help not only for theoretical and practical experts engaged in laboratory work but for clinicians and medical students as well.

K. JOBST

Herbert Lippert: *SI-Einheiten in der Medizin. Einführung in das Internationale Einheitensystem, Umrechnungstabellen, Normalbereiche.* Re-written by H. J. Raderecht and A. Gabler. VEB Gustav Fischer Verlag, Jena, 1980, 222 pages.

The book compiled by H. Lippert of Hannover had first appeared in 1978 and now has been rewritten by the authors, with special regard to the decrees valid in DDR. Only Chapter 4 of the book in an exception of this.

Chapter 1 expounds the history of the development of measuring systems by means of the theory of measuring and unit systems. Chapter 2 summarizes the characteristics of SI system (basic-supplementary-derived units, way of writing). Here, the authors treat the problems that must be solved in medicine during the change-over, detailing them for different branches of medicine.

The most important, most original chapters of the book are the third and the fourth ones. Here, one can find the conversion factor of almost every substance, enzyme, physical, chemical, biological constants occurring in medical practice. In addition the various components, and constants can be directly read off in SI units from tables constructed in a fashion similar to logarithmic tables. Within the tables a grey background denotes the normal range, and a light-grey one the limit-value range. The conversion factors are not just presented but also their mode of application demonstrated by examples. The tabulation of the values is unique among the publications issued on SI up to now.

Chapter 5 contains the table of relative atomic and molecular masses, the sex-dependent normal and limit-value range of the standard methods used in medical practice the conversion factor of substances and molecules rarely occurring in biology.

W. D. Arnold, U. Langheine: *Temperatureffekte an chirurgischen Metallimplanten bei den gebrauchlichen Elektrotherapieverfahren* (Temperature effects of electrotherapeutical methods on surgical metal implants) Fortschritte der experimentellen und theoretischen Biophysik, Band 24. VEB Georg Thieme Verlag, Leipzig, 1979 (in German).

Up to now, temperature effects of physicotherapeutical methods on the surround of surgical metal implants have been examined in phantom experiments only. However, in living organisms, the eliminating heat transport of blood circulation can also be of significance. There is no unequivocal opinion in the international literature on the extent of the tissular damage caused by different electrotherapeutical methods around metal implants in living organisms. At the same time owing to the development of bone surgery and metallurgy, more and more metallic foreign bodies were introduced into human organism during the last few decades (fixation of fractures, arthrodeses, implantation of artificial joints). It has been the aim of the authors to examine in animal experiments the damaging effect of heat produced around metal implants by the most frequently used electrotherapeutical methods.

Thin AO plates with 3–4 holes used in the treatment of human fractures were implanted in the thigh bones and Rush-nails placed in the thigh bones the medullary cavity of rabbits. The metals were stainless Cr–Ni–Mo alloys, also used in human treatment. Thermistores of type TNM 270 were soldered to implants; thus the heat effects could accurately be estimated in living organism.

The Chapters of the book deal with low-, long- and middle-frequency stimulations with shortwave, microwave, ultrasound, diathermic direct and alternating current electrotherapy. As known the physicochemical properties of collagen heated to a temperature

of 62 to 65 °C undergo changes and the collagon shrinks. This temperature range can thus be considered as a limit, at which living tissues will be damaged irreversibly.

The authors achieved damaging heat effects with short wave irradiations only (Kondensatorfeldmethode, Spulenfeldmethode), while microwaves, ultrasound, low frequency currents (Träbert's Reizstrom) all were ineffective. Thus, the latter ones can safely applied therapeutically in organs with metal implants in.

The authors treat the different electrotherapeutical methods in separate chapters each. They give detailed information on the quantity and quality of the influence as well as on the mode of recording the heat effects obtained.

The book may of use not only for biophysicists but also for orthopaedists, traumatologists and experts in physicotherapy, for it gives information on both the usefulness and the damaging heat effects of a therapeutical method of increasing importance and often applied after fixation of fractures with metals.

M. Forgon

Immunreaktionen in der Histochemie, by Luppa H. (ed.). Papers of the Joint Session of the Society of Topochemistry and Electron Microscopy of DDR and the Polish Society of Histochemistry and Cytochemistry held at Leipzig, September 1978. Acta histochemica, Vol. 22. VEB Gustav Fischer Verlag, Jena, 1980.

This supplement volume of Acta histochemica presents the lectures of the Joint Session of the Society of Topochemistry and Electron Microscopy of DDR and the Polish Society of Histochemistry and Cytochemistry held at Leipzig in 1978 with international participants from Austria, Bulgaria, Czechoslovakia, Finland, France, FRG, Hungary, Switzerland and the Soviet Union. The 40 papers published are arranged into five groups under the following titles:

Fundamentals of antibody formation (3 papers). These review articles summarize our present ideas on the biosynthesis of immunoglobulins, the structure of antibodies, their combining sites as well as on the antigenic determinants of globular proteins and the problems of humoral immune response.

Fundamentals of immunohistochemical techniques (8 papers). Besides reviewing conventional light- and electron microscopic methods of immunohistochemistry these papers deal with the problem of unspecific binding of antibodies to tissues and with the possibilities to prevent this pitfall of the method. Comparison of the efficiency of various immunoperoxidase methods, presentation of original ideas to preserve biological activities of cell substances during fixation with the use of bifunctional reagents, and approaches to quantitate immunocytochemistry make this part of the volume worth to study.

Lectins as antibody-analogs (5 papers). In this chapter, the use of lectins as antibody analogues in light- and electron microscopic study of carbohydrates, and different methods used for detection of lectin receptors on cell surfaces are discussed.

Immunohistochemical localization of hormones, enzymes and cell components (19 papers). About two third of the volume is dedicated to the immunohistochemical localization of hormones, enzymes and various cell constituents. Among the papers a review article gives up-to-date information about our 1978' knowledge on the peptide hormones of the brain and pituitary gland. This chapter contains very informative papers on the immunohistology of the cells in the anterior pituitary of mammals and non-mammalian vertebrates, of neurophysins, enteral hormones and cells in the islets of Langerhans. Reports on the immunohistological detection of ribosomes, antigammaglobulin factors of the serum, tissue-bound rheumatoid factor, an epidermal SH-protease inhibitor, plant viruses, characterization of antibodies against smooth muscle tissue, together with studies on antibody formation and on the effect of antibrain antibodies on the enzyme activity of the brain complete the list of topics covered by this chapter. Elegant studies deal with the application of protein A-gold complex for postembedding staining of intracellular antigens as well as demonstration of the use of the mixed-aggregation immunocytochemical method (MAGIC).

Miscellaneous (5 papers). This group of papers deals with studies by immunohistochemical techniques of the collagen, pancre-

atic enzymes, magnocellular hypothalamic nuclei, anaphylactic reactions and negative charges of the cell surface.

To sum up, this volume is a useful collection of studies demonstrating the merit of immunohistological methods in solving biological problems.

G. SÉTÁLÓ

Seminar Physik (Biophysik). W. Beier, K. Dähnert. VEB Georg Thieme, Leipzig 1980. 165 pages, 99 figures. 23.— MDN.

The system of university education in the GDR makes it compulsory that physics/biophysics be learned by the students of human and veterinary medicine, agriculture, biosciences and of many other branches of applied biology. The present booklet was written with the aim to help students and tutors perform their common work. The book consists of 32 tutorial topics. They are arranged in accordance with the classical scheme of physics: the system of units (1 chapter), mechanics (7 chapters), energetics (4 chapters), electricity (6 chapters), optics (7 chapters) and atomic physics (7 chapters) are collected in 32 chapters. Each one has the same structure: first the basic rules and formulae are summarized, then a few problems to be solved are presented. This is followed by a guide showing the way of how to solve the problems, and even references are given as to what formula has actually to be used. Finally, the results of the problems are listed.

The reader is somewhat puzzled as to the real aim of this book, though the authors try to make it clear. As explained in the preface a successful seminar requires both the students and tutors to be thoroughly prepared for the seminar and this book can help them to do so. However, one has the impression that the book gives too much help for the students and might prevent them from creative thinking. On the other hand, it seems too superficial for the tutors. Nevertheless, it may serve as a good suggestion of how to standardize the teaching program of seminars. Therefore, it is worthwhile to read this correct and clearly designed booklet whenever someone wants to become more familiar with the way of how physics and biophysics are taught in seminars for students of medicine and biology at the universities of the GDR.

T. LAKATOS

Struktur und Synthese von Immunglobulinen und die Bedeutung von Mediatoren bei Immunreaktionen by R. Dargel, D. Dettmer, (Ergebnisse der experimentellen Medizin, Bd. 33.) VEB Verlag Volk und Gesundheit, Berlin, 1979. 103 pages.

Recent examinations have revealed that, similarly to the immune response transmitted by cells, also soluble factors take part in the regulation of the humoral immune reactions. Thus, solute mediators also play a role in the immune responses during the cooperation of the thymus and the bone marrow depending lymphocytes. But the cellular cooperation figures not only in antibody synthesis but also in its inhibition; the release of soluble factors and mediators was demonstrated also in the latter case. Nowadays, also the effect of macrophages on humoral immune response has been brought into connection with mediators. The examination of the structure of immunoglobulins cannot be considered as finished. Further data on the connection between their structure and function can be expected from modern methodology. In the ten chapters of the book the above summarized new directions of immunology are reported by experts of international reputation, partly on the basis of their own investigations. (Antibody structure and immunological specificity, IgA-, IgM structure, regulation of antibody synthesis, interaction of macrophages and T cells, lymphokinins, transfer factor).

The booklet can lay claim to the interest of immunologists and biochemists.

K. JOBST

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A New Route to Nucleoside 5'-triphosphates

(Short Communication)

J. LUDWIG

Institute of Biophysics, Biological Research Centre, Hungarian Academy of Sciences, Szeged, Hungary

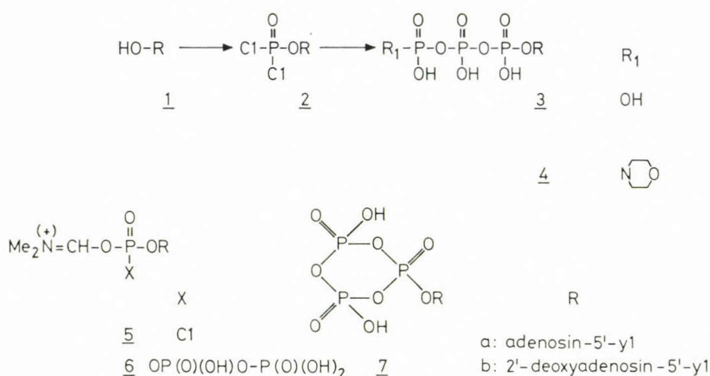
(Received October 9, 1981)

The Yoskihawa reaction, i. e. phosphorylation of unblocked nucleosides *(1)* with $POCl_3$ in trialkyl phosphates gives predominantly nucleoside 5'-phosphorodichloridates *(2)* (Yoshikawa et al. 1969). Subsequent *in situ* neutral (acidic) or alkaline hydrolysis, ammonolysis or alcoholysis of compounds *2* results in the formation of nucleoside *5*'-phosphates (Yoskihawa et al., 1969; Slotin, 1977) or nucleoside *3*',*5*'-cyciic phosphates (Tazawa et al., 1972) nucleoside *5*'-phosphorodiamidates (Simoncsits, Tomasz, 1975; Bottka, Tomasz, 1979) or nucleoside *5*'-alkylphosphates (Kim, Rosowsky, 1979).

It was found that compounds *2* can be transformed also into nucleoside 5'-triphosphates (*3*) by a short treatment performed *in situ* with an excess of tri--n-butylammonium pyrophosphate in DMF under anhydrous conditions followed by neutral hydrolysis, as shown on the examples of adenosine 5'-triphosphate (*3a*) and 2'-deoxyadenosine 5'-triphosphate (*3b*).

The reaction may proceed *via* the highly reactive imidoyl phosphate (*5* and *6*) and trimetaphosphate (*7*) intermediates. This supposition is based on the following observations. Phosphorodichloridates and DMF immediately form imidoyl phosphates which in turn react with phosphates to give pyrophosphates (Cramer, Winter, 1961). Trimetaphosphates *7* are readily formed from nucleoside 5'-(γ-imidoyl) triphosphate derivatives by intramolecular condensation (Glonek et al., 1974; Knorre et al., 1976; Webb, 1980). On this basis it seems reasonable to suppose that nucleoside 5'-(α-imidoyl) triphosphates (*6*) behave similarly. The γ-morpholidate *4a* was thus formed instead of *3a* when morpholine was added to the reaction mixture instead of aqueous $Et_3N \cdot H_2CO_3$. (Compound *4a* was previously described by Wehrli et al. (1965).

$POCl_3$ (0.26 mmol) was pipetted into a suspension of adenosine (*1a*, 0.20 mmol) in dry $(MeO)_3PO$ (0.5 ml) and the mixture was stirred at 0° for 1.5 h. A mixture of 0.5 M bis-tri-n-butylammonium pyrophosphate in anhydrous DMF (2 ml) and Bu_3N (0.2 ml) was quickly added under vigorous stirring. [The preparation of 0.5 M bis-tri-n-butylammonium pyrophosphate was carried out according to Moffat et al. (1964). By omitting the additional quantity of Bu_3N the yield significantly decreases.] After 1 min 1 M aqueous $Et_3N \cdot H_2CO_3$, pH = 7.5, was poured into the solution. After evaporation the residue was separated on a DEAE



cellulose [HCO_3^- form] column with a linear gradient of aqueous $Et_3N \cdot H_2O_3$, pH = 7.5. Yield: 86% of TLC pure *3a*, $R_f^A = 0.40$, $R_f^B = 0.29$. (Thin-layer chromatography was carried out on cellulose in a nPrOH: cc · $NH_4OH : H_2O$ = = 11/7/2 (A) and on PEI-cellulose in 1.5 M NaCl (B). Adenine: $P_{total} = 1.00 : 2.98$.

3b was prepared in exactly the same manner except that the phosphorylation was performed at −20° for 2 h. Yield: 78%, $R_f^A = 0.38$, $R_f^B = 0.25$; adenine: $P_{total} = 1.00 : 2.96$.

The simplicity, shortness and the possibility of using unlocked nucleoside as starting material (instead of 5'-nucleotide) render the method more advantageous than the earlier described procedures (e.g. anion-exchange, (Michelson, 1964) morpholidate (Moffatt, Khorana, 1961) and imidazolidate method (Hoard, Ott, 1965). The non-selectivity of the Yoshikawa reaction (Bottka, Tomasz, 1979; Dawson et al., 1977) (which depends on the nature of the heterobase) may cause the contamination of deoxyribonucleoside 5'-triphosphates with the isomeric 3'-triphosphates. In the case of *1b* this side product was formed to less than 0.5%. On the other hand ribonucleoside 2'(3')-triphosphates are unstable compounds and readily decompose to ribonucleoside 2',3'-cyclic phosphates (Khorana, 1961).

After the preparation of this manuscript a similar approach was described for the synthesis of several thymidine and 2'-deoxycytidine analogue 5'-triphosphates (Ruth, Cheng, 1981).

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The purification of Polynucleotide Phosphorylase from *Thermus aquaticus* by the use of Heparin-Sepharose 4B Affinity Chromatography

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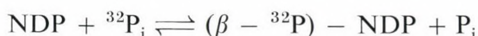
The polynucleotide phosphorylase of *Thermus aquaticus* was purified using ammonium sulfate fractionation and column chromatography on DEAE-cellulose, heparin-Sepharose 4B and DEAE-Sephadex A25. The enzyme was purified 1500-fold and was 90—95% homogeneous as checked by polyacrylamide gel electrophoresis. It has a molecular weight of 275 000 and consists of four identical subunits. The K_m values for the enzyme as determined in polymerization (ADP, GDP, UDP) and phosphorolytic reactions (polyA, polyU) are in the same concentration range as in the case of the enzyme deriving from mesophilic microorganisms. Furthermore, the enzyme is primer dependent and its activity is lost gradually at temperatures higher than 65 °C. In the base ratio of the copolymers followed the input base ratio polymerization reactions with polyUA, while with polyAG and polyUG a marked difference between the initial base ratio and the base composition of copolymers was observed.

Introduction

Polynucleotide phosphorylase (E.C.2.7.7.8 nucleoside-diphosphate: polynucleotide nucleotidyltransferase/catalyses the polymerization of ribonucleoside disphosphates and backwards the phosphorolytic cleavage of polynucleotides as well as an exchange between inorganic phosphate and β-phosphate or ribonucleoside diphosphates as summarized by the following equations (Grunberg-Manago, 1956):

$$\text{n NDP} \rightleftharpoons (\text{NMP})_n + \text{nP}_i$$



$$\text{NDP} + {}^{32}\text{P}_i \rightleftharpoons (\beta - {}^{32}\text{P}) - \text{NDP} + \text{P}_i$$

The enzyme has been found in every bacterium assayed so far (Godefroy-Colburn, Grunberg-Manago, 1972). It catalyzes the polymerization of ribonucleoside diphosphates which have an *anti* ribose-purine or pyrimidine ring conformation

Abbreviations: PNP-ase, polynucleotide phosphorylase; PAGE, polyacrylamide gel electrophoresis; SDS, sodiumdodecylsulphate; NMP, nucleoside-5'-monophosphate; polyA, polyadenylic acid; polyU, polyuridylic acid; polyUG, polyAG, polyAU, polyribonucleotide copolymers; DTT, dithiothreitol; PEI, polyethyleneimine

(Godefroy-Colburn, Grunberg-Manago, 1972). The ribonucleoside diphosphates with *syn* conformation (Kapuler et al., 1970) and the polynucleotides with phosphorylated 3'OH end (Grunberg-Manago, 1953) or with multistranded structure (Singer, 1958) inhibit the activity of the enzyme. Thus, polyguanylic acid with its stable four-stranded structure inhibits the PNP-ases of mesophilic microorganisms. However, at elevated temperatures, in consequence of its loosened structure, polyG can be synthesized with good yield (Thang et al., 1968; Thang, Grunberg-Manago, 1965; Kikuchi et al., 1977; Wood, Hutchison, 1976; Hishinuma et al., 1977).

Recently, PNP-ase from the thermophilic bacteria, *Thermus thermophilus* and *Bacillus stearothermophilus* was purified and well characterized (Kikuchi et al., 1977; Wood, Hutchison, 1976). Wood and Hutchison (1976) purified the *Thermus aquaticus* PNP-ase 31-fold and found the optimal temperature of the enzyme to be 72 °C.

In this work we describe a method for the purification *of Thermus aquaticus* PNP-ase. Some properties and characterization of the enzyme are also presented. In addition, the ability of the enzyme to synthesize polynucleotides containing guanylic acid and having well defined base composition will be demonstrated.

Materials and methods

Thermus aquaticus (ATCC 27 7237) was cultured as described by Heinen 1971) at 72 °C, using 0.2% (v/v) glycerol as carbon source. The specific activity of PNP-ase was constant throughout the fermentation, therefore, harvesting in the stationary phase yielded maximal amount of the enzyme.

Polymerization: The reaction mixture contained in a total volume of 100 μl, 0.1 M Tris-HCl, pH 9.0, 0.01 M $MgCl_2$, 0.01 M (^{14}C)-ADP or (^{14}C)-GDP (both 0.5 μCi/μmol), 10 μM oligoA (with respect to the mononucleotide), 0.005 M dithiothreitol, and the appropriate enzyme fraction. Incubation was at 65 °C. Following incubation, 10 μl of the reaction mixture were spotted onto polyethyleneimine (PEI)-cellulose TLC sheet (Macherey-Nagel, Düren, FRG) which was developed thereafter with 1.5 M KH_2PO_4, pH 3.4, solution. The chromatogram was dried, the start area cut out and its radioactivity counted by liquid scintillation.

Phosphorylation: In a total volume of 100 μl, the reaction mixture contained 0.1 M Tris-HCl, pH 8.2, 0.005 M DTT, 0.001 M $MgCl_2$, 20 μM polyribonucleotide (with respect to the mononucleotide), 0.01 M (^{32}P)-orthophosphate (1 μCi/μmol), pH 8.2. Incubation with the enzyme was at 65 °C. Ten μl of the reaction mixture was spotted onto PEI-cellulose sheet and chromatographed with 0.75 M KH_2PO_4, pH 3.4, for purine nucleotides, and with 0.3 M KH_2PO_4, pH 3.4, for pyrimidine nucleotides. The sheets were dried and autoradiographed overnight. The area of the appropriate nucleoside diphosphates was cut out and its radioactivity counted in water using the Cerenkov effect.

Determination of the copolymerization ratio using (^{3}H)-(^{14}C) *double labelling:* Under the conditions of the polymerization reaction in a total volume of 1.25 ml, copolymers were synthesized using DEAE-cellulose-chromatographed enzyme fraction (0.37 mg protein/ml) and (^{3}H) and (^{14}C) labelled nucleoside diphosphates as substrates. The samples were incubated at 65 °C for 3 h. Thereafter, the reaction mixture was cooled and extracted with one-tenth volume of phenol (1 M sodium acetate pH 4.5, (4 : 1 v/v) solution. The aqueous phase was sucked off and mixed with 2.5 volumes of 96% ethanol and kept at −40° for 12 h. The precipitate formed was dissolved and dialyzed exhaustively against distilled water. The radioactivity of the dialyzed solution was counted and the (^{3}H)/(^{14}C)-ratio calculated.

Protein was determined according to Spector (1978) using bovine serum albumin as standard. Heparin-Sepharose 4B was synthesized by using the method of Waldman et al. (1975). SDS-PAGE was made according to Weber, Osborn (1969), and isoelectric focusing as described by Wrigley (1971). The oligoA primer was synthesized by using the method of Wood, Hutchison (1976). The (^{3}H) and (^{14}C) labelled nucleoside diphosphates were purchased from Radiochemical Centre (Amersham), (^{32}P)-orthophosphate was a product of the Isotope Institute of the Hungarian Academy of Sciences.

Results

Purification of PNP-ase. Step I. Frozen *Thermus aquaticus* cells (105 g) were ground with 260 g of Al_2O_3 (Type 301 Sigma) in 180 ml 0.01 M Tris-HCl, pH 7.8, 0.01 M $MgCl_2$, 0.007 M β-mercaptoethanol solution (buffer A). The suspension was centrifuged at 5600 × *g* for 20 min and the precipitate was extracted with 50 ml of buffer A and centrifuged again. The pooled supernatant was spun down at 160 000 × *g* for 120 min. To the supernatant streptomycin sulfate dissolved in buffer A was added to a final concentration of 1.5%. The precipitate formed was discarded.

Ammonium sulfate fractionation. Step II. To the supernatant of Step I powdered ammonium sulfate was added to 35% saturation at 4 °C, the pH was adjusted to 7.0. The solution was stirred for half an hour and centrifuged at 16 000 × *g* for 20 min. The supernatant was brought to 55% saturation with ammonium sulfate as described above. The precipitate was dissolved in buffer A and dialyzed against the same solution.

DEAE-cellulose chromatography. Step III. The dialyzed enzyme solution was diluted three-fold with buffer A and applied onto a Whatman DE 52 column (3 × 21 cm) equilibrated with buffer A. The column was washed with 200 ml of buffer A and eluted with a 0.0 to 0.5 linear gradient of KCl in buffer A (250 − 250 ml). Ten ml fractions were collected and their phosphoroyltic activity was assayed. The active fractions were pooled and dialyzed against 0.1 M Tris-HCl, pH 8.2, 0.001 M $MgCl_2$, 0.007 M β-mercaptoethanol buffer (buffer B).

Heparin-Sepharose 4B chromatography. Step IV. The dialyzed enzyme solution was applied onto a heparin-Sepharose 4B column (1.4 × 33 cm) equilibrated with buffer B. This step was made at 40 °C because at lower temperature the enzyme would not bind to the gel. Earlier attempts to use Cibarcon Blue-Sepharose column failed because the enzyme was bound so tightly to this type of column that we were unable to elute it even at a very high ionic strength (1 M NaCl and 20 mM ADP, results not shown). The bound enzyme was eluted from the gel with a linear KCl gradient (0.0 to 0.5 M, 100-100 ml) at 4 °C. Four ml fractions were collected and their phosphorolytic activity and protein composition (PAGE) were checked. The less contaminated and highly active fractions were pooled and dialyzed against 0.05 M Tris-HCl, pH 7.8, 0.002 M $MgCl_2$, 0.007 M β-mercaptoethanol buffer (buffer C).

DEAE-Sephadex A25 chromatography. Step V. The enzyme was further purified on a DEAE-Sephadex A25 column (1.4 × 6.5 cm) equilibrated with buffer C. The enzyme was eluted with a linear KCl gradient (0.0 to 0.5 M, 75–75 ml) in buffer C at 4 °C. Fractions of 2 ml were collected and their phosphorolytic activity was determined. Active fractions were pooled and vacuum dialyzed.

The purification steps are summarized in Table 1. The enzyme was purified 1500-fold, based on its polymerizing activity. The extent of purification was lower when the calculation was based on the phosphorolytic assay. Measurement of enzyme activity in the crude extract is relatively inaccurate, since disturbing enzymes are present in the enzyme preparation. Our enzyme preparation was at least 90–95% homogeneous as judged by PAGE (Fig. 1).

Properties of Thermus aquaticus PNP-ase. We have determined the molecular weight of the purified *Thermus aquaticus* PNP-ase using Sephadex G200 molecular sieving. Figure 2 shows that the molecular weight is 275 000. When the subunit structure of the enzyme was examined by SDS-PAGE, we found that only one type of subunits with a molecular weight of 70 000 was present (Fig. 3). The above

Table 1

Purification of Thermus aquaticus PNP-ase

Steps of purification	Phosphorolytic activity	Polymerizing activity	Protein conc.	Volume	Purification (fold)*	Yield %
	$\frac{\text{nmol ADP}}{\text{mg prot} \times \text{min}}$	$\frac{\text{nmol GDP}}{\text{mg} \cdot \text{prot} \times \text{min}}$	$mg \times ml^{-1}$	ml	—	—
I	3.67	0.52	11.2	250	1	100
II	6.69	0.97	7.75	175	1.86	85.6
III	34.3	6.2	3.77	75	11.9	120.2
IV	712	116	0.06	48	223	22.9
V	4097	818	0.19	1	1573	10.9

* Based on the polymerizing activity

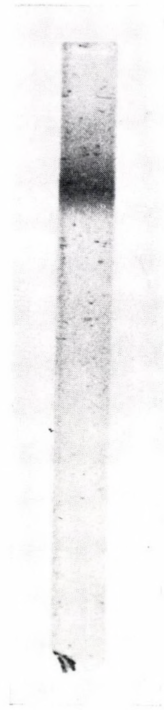


Fig. 1. The PAGE pattern of purified *Thermus aquaticus* PNP-ase. The gel was loaded with 26 μg protein from step V of purification and stained with amidoblack

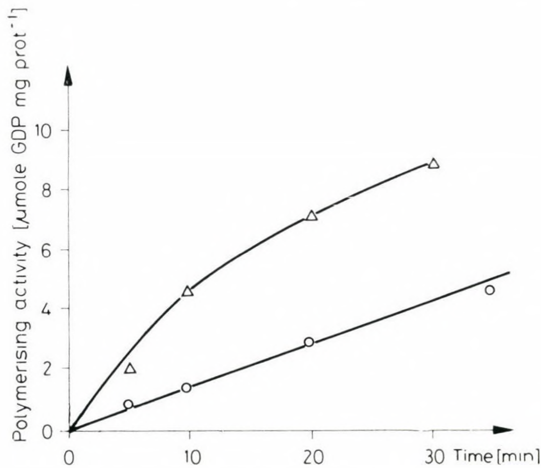


Fig. 2. Primer dependence of *Thermus aquaticus* PNP-ase. Effect of 10 μM oligoA on the polymerizing activity of the purified enzyme (28.5 μg/ml). —△—△—, PNP-ase activity in the presence of primer; —○—○—, PNP-ase activity without the primer

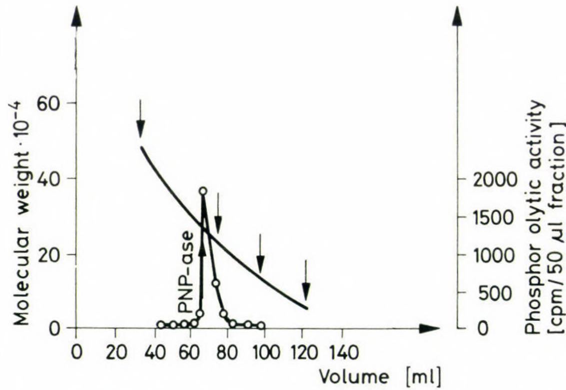


Fig. 3. Determination of the molecular weight of the purified enzyme by Sephadex G200 molecular sieving. One ml of purified enzyme was applied onto a Sephadex G200 column (1.2 × 55 cm) and eluted with 0.05 M Tris-HCl, pH 8.2, 0.002 M $MgCl_2$, 0.007 Mβ-mercapto-ethanol buffer at 4°C. Four ml fractions were collected and their phosphorolytic activity was determined. The proteins used as markers were: Ferritin 475 000, catalase 235 000, aldolase 145 000, albumin 68 000

data indicated that the enzyme consists of four identical subunits. Isoelectric focusing in polyacrylamide gel showed the isoelectric point of the pure enzyme to be 4.3.

As to the other characteristics of the enzyme, the effect of supplementing the reaction mixture with a primer is shown in Fig. 4. Ten μM of oligoA, as a primer, was used in the standard polymerizing reaction mixture. The enzyme activity

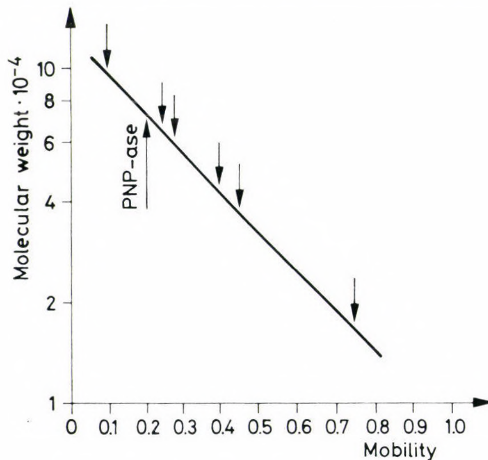


Fig. 4. Determination of the subunit structure of the purified PNP-ase by SDS-PAGE. The enzyme was denatured by heating at 100°C for 5 min in 1% SDS, 100 mM β-mercapto-ethanol. Protein markers were: phosphorylase-a 94 000, albumin 68 000, catalase 60 000, aldolase 40 000, glycerolaldehydephosphate dehydrogenase 36 000, myoglobin 17 200

Table 2

*K_m values for purified Thermus aquaticus PNP-ase**

Type of reaction	K_m value
Polymerization	ADP 4.7 mM GDP 3.8 mM UDP 3.9 mM
Phosphorolysis**	polyA 4.0 μM (with respect to mononucleotide) polyU 8.0 μM (with respect to mononucleotide)

* K_m values were determined by using Lineweaver-Burk plots

** The polynucleotides contained more than 250 mononucleotide residues

assayed in the presence of the primer was always higher than in its absence during the incubation period tested (40 min). The K_m values for the purified PNP-ase both in the phosphorolytic and polymerizing reactions were also determined (Table 2).

To study the thermal stability of the enzyme purified from *Thermus aquaticus* it was incubated in the phosphorolytic reaction mixture at different temperatures. Figure 5 shows that the enzyme looses its phosphorolytic activity at temperatures higher than 65 °C.

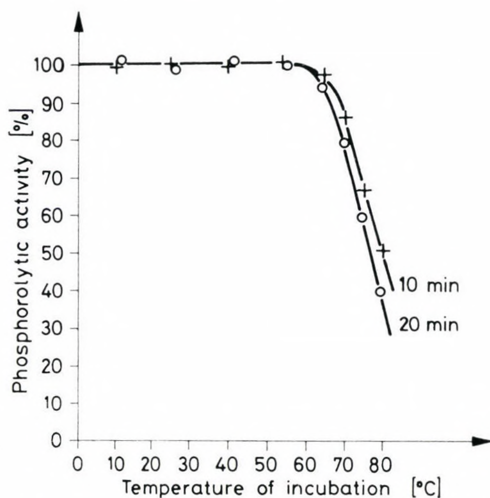


Fig. 5. Heat stability of the PNP-ase. The enzyme (from step IV, 15 μg/ml) was incubated in the phosphorolytic reaction mixture at different temperatures for 10 or 20 minutes. Afte r incubation the mixtures were supplemented with poly A and (^{32}P)-orthophosphate and their phosphorolytic activity was determined at 65°C. — + —, 10 min incubation; — ○ —, 20 min incubation

We have also measured the copolymerizing activity of *Thermus aquaticus* PNP-ase with different pairs of nucleoside-diphosphates as substrates by using the (^{3}H)/(^{14}C) double labelling technique. In these experiments the combined substrate concentrations were kept constant, while the ratio of the two nucleotide components was changed. No primer was included. Figuie 6 demonstrates that in the case of polyUA no difference could be observed between the initial base composition of the reaction mixture and the base composition of the products, while in the case of polyUG and polyAG the composition did not follow the set base ratio.

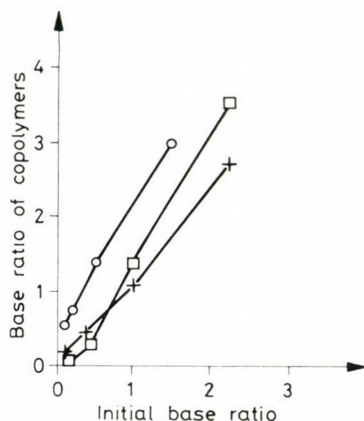


Fig. 6. Copolymerizing activity of *Thermus aquaticus* PNP-ase. In this experiment DEAE-cellulose-chromatographed enzyme was used (0.37 mg protein/ml). The total substrate concentration was 0 04 M. Incubation was at 65°C for 180 min. The primer was left out from the reaction mixture. —+—, [5-^{3}H)UDP and (U-^{14}C)ADP]polyUA; —○—, [(5-^{3}H)UDP and (U-^{14}C)GDP]polyUG; —□—, [(2-^{3}H)ADP and (U-^{14}C)GDP]polyAG

Discussion

The polynucleotide phosphorylase of *Thermus aquaticus* was purified 1500-fold and proved to be at least 90–95% homogeneous as judged by PAGE. During purification heparin-Sepharose 4B affinity column was used for the first time in the purification of PNP-ases. Heparin is a polysaccharide with many negatively charged groups, similarly to polynucleotides. Heparin itself, at 1 mg/ml (approx 0.1 mM) concentration decreases the phosphorolytic activity by 70% (unpublished result). The enzyme binds to the gel only at temperatures higher than 40 °C, perhaps as a consequence of a favourable change of protein conformation.

The size and subunit structure of PNP-ase from different microorganisms differ from each other. The enzyme isolated from *Escherichia coli B* has a molecular weight of 200 0000 and has two subunits of 95 000 each (Lechrach et al., 1971). The enzyme from *Bacillus stearothermophilus* consists of four 51 000 subunits (Wood, Hutchison, 1976). The *Thermus thermophilus* enzyme is a trimer

with subunits of 25 000, 13 000 and 95 000 daltons (Hishinuma et al., 1977). The *Thermus aquaticus* enzyme, as shown in this paper, is composed of four identical subunits.

Concerning the primer dependence, different strains exhibit a wide variety. While *Escherichia coli B* (Godefroy et al., 1972) and *Thermus thermophilus* (Hishinuma et al., 1977) PNP-ases are primer dependent, the *Micrococcus lutens* enzyme is primer independent (Klee, 1967). In the latter case, however, tryptic digestion transformed the enzyme into a primer dependent form (Mose, Singer, 1970). According to our experiments the *Thermus aquaticus* PNP-ase is primer dependent.

We also determined the K_m values for *Thermus aquaticus* PNP-ase in both the phosphorolytic and polymerizing reactions. The K_m values are in the range found for the enzymes from mesophilic strains with the exception that GDP is the normal substrate of the thermophilic enzyme. On the other hand, the enzyme was unable to degrade polyG in the phosphorolytic reaction (data not shown).

The pH of the enzyme is 4.3 which is quite similar to the values found for other PNP-ases from different sources.

Our enzyme was shown to loose its activity at temperatures higher than 65 °C, whereas Wood and Hutchison (1976) found the optimal temperature of the enzyme to be 72 °C, which is equal to the temperature optimum of growth. The loss of activity in our case might have been due to the low protein concentrations in our tests.

Examining the copolymerizing reactions Grunberg-Manago (1953) found that the base ratio of copolymers was equal to the initial base ratio. Farber and Chargaff (1967) took the opposite standpoint. When they copolymerized ADP and CDP the polymer contained always more cytidylic acid than adenylic acid.

In the case of *Bacillus stearothermophilus* the enzyme favoured GDP to UDP (Wood, Hutchison, 1976). With the technique of double isotope labelling we examined the *Thermus aquaticus* PNP-ase in copolymerization reactions. In the case of polyUA the base ratio of the polymer was the same as in the initial reaction mixture; but in the case of polyAG and polyUG the base ratio of the product and that of the initial reaction mixture were different, but well definable.

Thus, *Thermus aquaticus* PNP-ase can be used to synthesize polymers containing guanylic acid with well defined base composition.

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Coenzyme Induced Changes in the Interdimeric Contact Surface of D-Glyceraldehyde-3-Phosphate-Dehydrogenase

(Short Communication)

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D-Glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate: NAD* oxidoreductase phosphorylating, E.C.1.2.1.12) is composed of four sequentially identical subunits, the tetrameric form of all known GAPD molecules has a molecular weight of 145 000 (Harris, Waters, 1976). The amino acid sequences of GAPD molecules from evolutionally distant species are unusually highly conserved, for example there is 68% identity in the amino acid sequences of the yeast and pig muscle enzymes (Jones, Harris, 1972). Despite this high degree of similarity between pig muscle and yeast enzymes, mammalian muscle GAPDs display negative cooperativity in coenzyme binding, whereas in the case of the yeast enzyme the binding of coenzymes shows positive cooperativity (Harris, Waters, 1976). Even though recent studies have revealed that the extent of cooperativity depends on the method used for purification of enzymes (Scheek, Slater, 1978; Scheek et al., 1979) and the experimental conditions (Reynolds, Dalziel, 1979) the differences in the allosteric properties of the yeast and mammalian enzymes appear real and suggest that the subunit contacts transmitting the cooperative changes probably differ in the two types of GAPD molecules (Harris, Waters, 1976). The finding of numerous authors that yeast and mammalian GAPDs readily hybridize (Osborne, Hollaway, 1974, 1975; Muronetz et al., 1979; Cherednikova et al., 1980; Scheek et al., 1980) seems to contradict this conclusion since the formation of hybrids shows that the heterologous subunit association is not significantly different from homol ogous association. To clarify this apparent contradiction we have investigated the hybridization of porcine muscle GAPD with yeast enzyme and the effect of different coenzyme and substrate combinations on the formation and stability of hetero- and homotetramers.

Four times recrystallized GAPD was prepared from pig skeletal muscle according to a described procedure (Elődi, Szörényi, 1956). Crystalline yeast GAPD was purchased from Boehringer Mannheim, Germany. Yeast GAPD was free of bound coenzyme. Both enzymes were gel-filtered on Sephadex G-25 columns (1 × 25 cm) equilibrated with the borate buffer used for hybridization (see below).

* *Abbrevation:* GADP, D-glyceraldehyde-3-phosphate dehydrogenase

The porcine enzyme thus prepared contained two firmly bound NADs as judged by the A_{280}/A_{260} ratio (Fox, Dandliker, 1956), since gel filtration in borate buffer removes two of the four NADs of porcine holo-GAPD, presumably through the complexation of NAD with borate (Smith, Johnson, 1976). NAD and NADH (Reanal, Budapest and Boehringer, Mannheim) were commercial products. Glyceraldehyde-3-phosphate was prepared from fructose-1,6-bisphosphate by the method of Szewczuk et al. (Szewczuk et al., 1961).

Hybridization of yeast and pig muscle GAPDs was carried out under conditions similar to those developed by Suzuki et al. (Suzuki et al., 1976). The hybridization mixture contained equimolar amounts of the two enzymes at a protein concentration of $0.3-1.5\times10^{-4}$ M in the 10 mM Tris–20 mM sodium borate–1 mM EDTA–1 mM mercaptoethanol, pH 7.5 buffer. The reaction mixture was incubated at 30 °C and aliquots were withdrawn at intervals and analyzed for hybrid by electrophoresis in 50 mM sodium phosphate-5 mM EDTA, pH 7.0 buffer on cellulose acetate as described by Suzuki et al. (1976). The ratio of hetero- to homotetramers did not depend on the duration of electrophoresis. No loss of enzyme activity occurred within the time scale of our experiments. The cellulose acetate strips were stained with Amido Black and scanned with a Telechrom S videodensitometer (Chinoin, Budapest).

The time course of hybrid formation during incubation of an equimolar mixture of yeast and porcine muscle GAPD is shown in Fig. 1. When equilibrium is attained approximately 30% of the protein is present in the band corresponding to hybrid. The position of the hybrid in the electrophoretograms shows that it is an equimolar mixture of yeast and porcine GAPD subunits, i.e. it corresponds to hybrid of the Y_2P_2 type that arises through the association of dimers of the two enzymes. In our experiments no evidence for the formation of the hybrid of the Y_3P or YP_3 type was obtained. The formation of significant amounts of Y_2P_2 hybrid indicates that the interdimeric contact surfaces of the yeast and pig GAPDs are very similar, permitting the realization of interdimeric contacts similar to those present within the homotetramers.

Addition of NADH to hybridization mixture after the equilibrium position characteristic of substrate-free system has been attained leads to the rapid disappearance of the hybrid (Fig. 1). Since less than 10% of the total protein remains at the position of hybrid in the presence of NADH, the binding energy of association across the interdimeric contact surface must be significantly lower in the NADH-hybrid than the binding energy of association in the NADH-homotetramer(s). In terms of contact surface this means that NADH induces substantial changes in the interdimeric contact surface regions where the yeast and pig enzymes are different, so that heterologous association can no longer compete with the homologous association.

In contrast with the effect of the reduced coenzyme, NAD addition to an equilibrium hybridization mixture fails to evoke the disappearance of the hybrid (Fig. 1). Since the addition of NAD to a hybridization mixture prior to hybrid formation prevents the appearance of hybrid, this indicates that equilibrium has

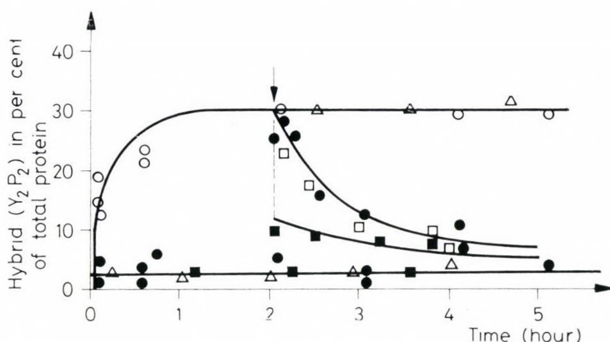

Fig. 1. Effect of various ligands on the formation and stability of the hybrid Y_2P_2. The hybridization mixture contained equimolar amounts of yeast and porcine GAPDs at a protein concentration of 5— 5-mg/ml in the 10 mM Tris-20 mM sodium borate — 1 mM EDTA — 1 mM mercaptoethanol, pH 7.5 buffer. The reaction mixture was incubated at 30°C and the amount of hybrid present was determined at intervals by cellulose acetate electrophoresis. Symbols: ○—○, no additions; △—△, 2 mM NAD; □—□, 1.85 mM NAD and 0.15 mM NADH; ■—■, 0.15 mM NADH; ●—●, 2 mM NAD and 2 mM GAP and 10 mM P_i, initial substrate concentrations. The arrow indicates the addition of ligands to preformed hybrid

not been attained within the time-scale of our experiments. NAD is known to increase the interdimeric binding energy in porcine GAPD (Smith, Johnson, 1976), therefore the fact that hybrid does not disappear in the presence of excess NAD indicates that the hybrid is also quite stable in the presence of this coenzyme. These findings are in agreement with the results of Osborne and Hollaway (1975) and Scheek et al. (1980), obtained in the case of hybridization of rabbit muscle and yeast enzymes. Nevertheless our experiments can not answer the question whether homotetramer or heterotetramer formation is favoured by NAD. In the case of the hybrid of rabbit muscle and yeast GAPD Scheek et al. (1980) demonstrated that excess NAD stabilizes the hybrid, but low concentration of NAD, equimolar to enzyme tetramer, causes hybrid decomposition, i.e. NAD also shifts the equilibrium to homotetramers.

Since during catalytic cycles of GAPD the enzyme interacts with NAD and NADH we studied the simultaneous effect of both coenzymes on the hetero-homotetramer equilibrium. As shown in Fig. 1, addition of 1.85 mM NAD and 0.15 mM NADH elicits the rapid breakdown of the hybrid, whereas NAD alone has not caused the disappearance of the heterotetramer. The dominance of the effect of NADH over that of NAD is even more surprising since GAPD displays weaker affinity for NADH than for NAD (von Ellenreider et al., 1972). Nevertheless because of cooperative phenomena in coenzyme binding, it is still impossible to predict whether in a mixture containing both reduced and oxidized coenzymes, some of the coenzyme binding sites will not be saturated by NADH. We checked this possibility by gel filtration of porcine GAPD on Sephadex G 50 (medium) columns (0.6 × 50 cm) equilibrated with hybridization buffer containing 1.85 mM

NAD and 0.15 mM NADH. The concentrations of protein, NAD and NADH in the elution profile were determined spectrophotometrically. Evaluation of the elution profiles according to the method of Hummel and Dreyer (1962), showed that 0.3 ± 0.2 mole of NADH was bound to GAPD per mole tetramer and 3.8 ± 0.4 NAD per mole tetramer. It thus appears that in mixtures containing both reduced and oxidized coenzymes NADH may compete with NAD for the forth binding site.

We suggest that the dominance of NADH over NAD is explained by assuming that NAD also favours homotetramer formation but the NAD induced increase in binding energy, in regions that are similar in yeast and pig enzymes, serve as large activation barriers not permitting the rapid breakdown of the hybrid. In the case of NADH this effect causing the tightening of heterotetramers is not observed, the activation barrier is low therefore rapid breakdown of hybrid can take place. In mixtures of NAD and NADH the effect of NADH may appear dominant since even transient binding of NADH may lower the activation energy of tetramer dissociation and facilitate the attainment of the thermodynamically more favourable state.

In the presence of an equilibrium mixture of substrates and coenzymes the disruption of hybrids proceeds in a manner similar to that observed in the presence of NAD and NADH mixed in a ratio similar to that found in the equilibrium mixture. Since in an equilibrium mixture of substrates GAPD exists largely in the form of the acylenzyme (Trentham, 1971), this indicates that acylation does not alter the tendency of the holoenzyme to prefer the homotetrameric state, neither does it significantly alter the activation barrier of dissociation of hybrid into dimers.

We have found that NADH addition to the hybridization mixture leads to disappearance of the hybrid. Similarly, in the presence of the oxidized coenzyme the homotetrameric state is favoured thermodynamically even though the breakdown of the hybrid, in the absence NADH, is slow. The results suggest that conversion of the apoenzyme to holoenzyme must be accompanied by significant changes in the interdimeric contact regions of the enzyme(s) bringing to surface structures that are markedly different in the pig and yeast enzymes so that heterologous association can no longer occur.

X-ray crystallography of lobster muscle GAPD have revealed only minor structural differences between the apo- and holoenzymes, the most significant conformational change upon NAD binding occurs at Trp-193 that is present in the S-shaped loop which is involved in the R axis related subunit–subunit interactions (Murthy et al., 1980). Similar studies on the apo- and NAD-holo-GAPD of *Bacillus stearothermophilus* have also been interpreted to mean that the S loop is the site of the most significant changes occurring during the apo-to holoenzyme transition (Biesecker et al., 1977). It is noteworthy in this respect that in the region of the S loop undergoing conformational change (residues 190 – 198) significant nonconservative amino acid differences exist between yeast and pig enzymes that include residues involved in subunit–subunit interactions across the R axis (Olsen

et al., 1975). The fact that the breakdown of hybrid is much faster in the presence of NADH than NAD points out that the reduced and oxidized coenzymes do not cause identical conformational changes at the interdimeric contact region. At present, however, no data are available on the structure of NADH-holo-GAPDs.

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The Effect of Dibromo-Dulcitol and Dianhydro-Dulcitol (Galactitol) on RNA Synthesis in Ascites Tumor Cells

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The mechanism of action on RNA synthesis of anticancer dibromo-dulcitol (DBD, NSC-104800) and dianhydro-dulcitol (DAD, or elsewhere dianhydrogalactitol, DAG, NSC-132313) was investigated. Rats, bearing Yoshida or Novikoff hepatoma ascites tumor cells sensitive to these drugs were treated with doses equivalent to half the LD_{50} value. Nucleolar RNA (noRNA) and nuclear RNA (nRNA) were pulse labelled with ^{3}H-uridine, isolated and fractionated on sucrose density gradient. After 18 h treatment with either drug and after 3 h with DAD noRNA synthesis increased and the rate of ribosomal RNA (rRNA) precursor processing was enhanced. Investigation of low-molecular weight nRNAs (LMW nRNAs) (separated by polyacrylamide gel electrophoresis) showed increased synthesis and/or accumulation of RNA species (5S RNA, uridylic acid rich RNAs) related to rRNA synthesis.

The tritium labelled drugs were bound to distinct fractions of nRNA, separated by sucrose density gradient ultracentrifugation, both *in vivo* and *in vitro*. This fact may be explained by the formation of intra-, or intermolecular crosslinking of pre-messenger RNA.

The enhanced RNA synthesis might be interpreted as an alteration in the functions of nuclear proteins, involved in the regulation of gene transcription and processing of RNA precursors.

Introduction

DBD and its diepoxy derivative, DAD are widely used anticancer agents (Sellei et al., 1969; Andrews et al., 1974; Mischler et al., 1979; Németh et al., 1972; Eagan et al., 1976; DeJager et al., 1977). Although, there are some indications that DAD is the active form *in vivo*, experimental and clinical studies revealed

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Abbreviations: BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethy)-3-cyclohexyl-1-nitrosourea; DBD, 1,6-dibromo-1,6-dideoxy-dulcitol or 1,6-dibromo-1,6-dideoxy-galactitol or NSC-104800 or Mitolactol^R; DAD, 1,2-5,6-dianhydro-dulcitol or 11,2-5,6-dianhydrogalactitol or diepoxy-dulcitol or NSC-132313; nRNA, nuclear RNA; noRNA, nucleolar RNA; LMW nRNA, low molecular weight nRNA; rRNA, ribosomal RNA; mRNA, messenger RNA; SDS, sodium dodecyl sulfate.

differences between these drugs (Németh et al., 1972; Horváth et al., 1979; Elson et al., 1968; Institóris et al., 1970). These differences may be due either to difference in transport (Institóris et al., 1973), to different alkylating properties or to the action of several intermediates produced from DBD (Belej et al., 1972; Horváth, Institóris, 1967).

Studies on nucleic acids consistently showed inhibition of precursor incorporation into DNA after DBD or DAD treatment, although to various extents in different types of cell studied (Hidvégi et al., 1967; Vályi-Nagy et al., 1969; Jeney et al., 1970; Börzsönyi et al., 1969; Hidvégi et al., 1976; Baló-Banga et al., 1975; Pályi, 1975). The inhibitory action on cell proliferation is currently regarded as a result of covalent binding of DBD or DAD to chromatin components (Institóris et al., 1974; Jeney et al., 1976a; Jeney et al., 1979). Experiments also demonstrated a reduction in the amount and synthesis of chromatin proteins and impaired interaction between DNA and histone HL (Jeney et al., 1970; Jeney et al., 1979).

Contrary to the unanimous response of DNA synthesis in various types of cell, the RNA synthesis either decreased, as in HeLa cells and bone marrow cells (Hidvégi et al., 1967) or increased, as in Yoshida cells (Vályi-Nagy et al., 1969) and in tonsillar cells (Hidvégi et al., 1976). A variable response in RNA synthesis to DBD treatment was discovered in P388 lymphoma cells which showed enhanced synthesis at an early growth stage and a reduced one at a later stage of growth (Jeney et al., 1976b).

The purpose of this study was to reveal the action of DBD and DAD on the synthesis of various types of RNA in early growth stage cells of sensitive tumors.

Materials and methods

Cells: Yoshida sarcoma ascites tumor and Novikoff hepatoma ascites tumor strains, both sensitive to alkylating agents were used 2–4 days after transplantation of 10*f* cells into 100–200 g CFY female rates.

Treatment: DBD (250 mg/kg, $LD_{50}/2$) was administered i.p. in 2% (v/v) Tween-80 suspension. DAD (7.5 mg/kg, $LD_{50}/2$) was dissolved in isotonic saline and given i.p. Control animals were treated in a similar way without drugs. DBD and DAD were produced by Chinoin Pharmaceutical and Chemical Works Ltd., Budapest, Hungary.

Radioactive labelling: 100 μCi (3.7 MBq)/100 g body weight ^{3}H-5-uridine (specific activity: 5 mCi (185 MBq)/mM, purchased from Radiochemical Centre, Amersham, England) was given i.p. in 1 ml isotonic saline. ^{3}H-DAD and ^{3}H-DBD were labelled at carbon position 1 of the hexitol moiety as reported previously (Institóris et al., 1973). The specific activity of ^{3}H-DBD of ^{3}H-DAD was 0.5 mCi (18.5 MBq)/mg).

Preparation of nuclei and nucleoli: The tumor cells were removed from the animals and washed twice with cold isotonic saline. Nuclei were isolated by homogenization in 0.5% (w/v) citric acid (Higashi et al., 1966), and centrifuged at 2000 × *g* for 15 min. The pellet was suspended and homogenized again in 0.5% (w/v) citric acid containing 0.25 M sucrose, layered on 0.5% citric acid containing 0.88 M sucrose and centrifuged. Nucleoli were isolated as described in detail by Muramatsu and Busch (1967). The quality of the nuclei and nucleoli was checked by phase contrast microscopy.

Isolation and analysis of labelled RNA: RNA was extracted from nuclei or nucleoli by homogenization in a Potter – Elvehjem tissue grinder with loosely fitted pestle in 0.05 M acetate buffer, pH 5.1, containing 0.14 M NaCl and 0.3% (w/v) SDS. An equal volume of phenol was added and this mixture stirred at 65 °C for 5 – 10 min. After centrifugation the phenol extraction was repeated and the RNA precipitated by the addition of 2.5 volumes of ethanol containing 2.5% (w/v) potassium acetate (Steele et al., 1965; Steele, Busch, 1967).

One mg RNA was fractionated by ultracentrifugation in a 10 – 45% (w/w) sucrose density gradient containing 0.1 M NaCl, 1 mM EDTA, 0.01 M acetate buffer, pH 5.1, in the SW 27 rotor of the Beckman Model L2-65B ultracentrifuge at 26 000 r.p.m. for 19 h, or in the ^{3}H-DAD and ^{3}H-DBD labelling experiments, for 12 h. The gradient was fractionated by an ISCO Model 640 fractionator. Absorbance at 254 nm was recorded continuously and the radioactivity of 1 ml fractions was measured according to Steele et al. (1965) in a Beckman LSC-100 or a Nuclear Chicago Mark III liquid scintillation spectrometer. The LMW-nRNA fractions were pooled, precipitated and further fractionated by polyacrylamide gel electrophoresis as described previously (Fónagy, Hidvégi, 1975).

The labelled whole nuclear RNA was separated by poly(U)-Sepharose 4B chromatography to poly(A)$^+$ and poly(A)$^-$ RNAs (Lindberg, Persson, 1972).

Results

Synthesis of nRNA and noRNA

nRNA synthesis was followed in Yoshida ascites cells treated *in vivo* with doses of DBD and DAD equivalent to half the LD_{58} values. ^{3}H-uridine was given i.p. for 20 min (short pulse) or for 90 mind before sacrificing the animals. Table 1 shows that after 18 h treatment both drugs caused an increase in the specific radioactivity of whole nRNA by 30 to 70% above the control. There was a more extensive increase using ^{3}H-uridine for 90 min than for 20 min. Treatment for 3 h enhanced the labelling of nRNA only with DAD but not with DBD.

The labelled nRNA was fractionated by sucrose density gradient ultracentrifugation. Figure 1 shows that in the control cells most of the label was detected in the rapidly sedimenting 45*S* – 65*S* RNA fractions after 20 min labelling. Three h treatment with DAD but not with DBD increased the label in the rapidly

Table 1

Specific radioactivities of ${}^{3}H$-uridine labelled RNAs

Labelled nRNA and noRNA of ascites tumor cells were isolated and their specific radioactivity measured as described in Methods. Specific radioactivity is expressed as cpm/$E_{254} \times 10^{-3}$. The specific radioactivities of 28*S* and 45*S* RNA fractions were calculated from sucrose density gradient pattern of nRNA after 90 min labelling. The values are the means of 3 to 4 experiments. The ratios of the specific radioactivities of 28*S* and 45*S* RNAs, the standard deviations and the percentage values with reference to the control (in paranthescs) are also given

Drug treatment	Labelling time: 20 min		Labelling time: 90 min			
	noRNA	nRNA	nRNA	45*S* RNA	28*S* RNA	28*S*/45*S*
None	7.5 ± 0.6 (100)	3.8 ± 0.3 (100)	2.7 ± 0.3 (100)	3.4 ± 0.3 (100)	1.1 ± 0.1 (100)	0.32
DBD, 3 h	8.7 ± 0.6 (116)	3 5 ± 0.3 (92)	3.0 ± 0.3 (111)	3 8 ± 0.4 (112)	1.7 ± 0.2 (155)	0.45
DBD, 18 h	10.2 ± 0.9 (136)	4.8 ± 0.4 (126)	4.6 ± 0.4 (170)	6.7 ± 0.5 (197)	4.6 ± 0.4 (418)	0.69
DAD, 3 h	9.9 ± 0.9 (132)	6.5 ± 0.5 (171)	4.6 ± 0.4 (170)	5.5 ± 0 4 (162)	4.8 ± 0.4 (436)	0.87
DAD, 18 h	9.6 ± 0.9 (128)	4 8 ± 0.4 (126)	4 0 ± 0.4 (148)	4.6 ± 0.4 (135)	4.1 ± 0.4 (372)	0.89

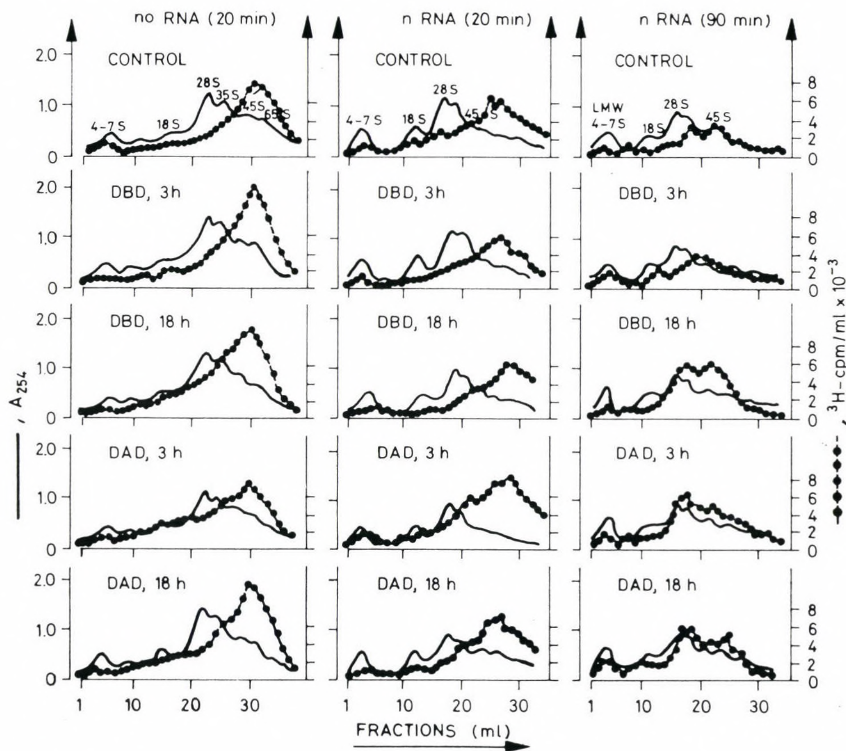

Fig. 1. Incorporation of ^{3}H-uridine into noRNA and nRNA. Rats bearing ascites tumor cells were treated with DBD or DAD for 3 h or 18 h. Twenty min or 90 min before the termination of the treatment with the hexitol 100 μCi (3.7 MBq)/100 g body weight ^{3}H-uridine was given i. p. The noRNA was isolated, purified, its specific activity determined and 1 mg was fractionated by sucrose density gradient centrifugation (10–45% (w/w) at 26 000 r. p. m. for 19 h). The solid line represents the optical density at 254 nm and the dotted line the radioactivity in 1 ml fractions. Sedimentation is from left to the right

sedimenting RNA fractions, in agreement with the specific radioactivity data of the whole nRNA (Table 1). Eighteen h treatment with either drug increased the specific activity by 50 to 80% in the rapidly sedimenting RNA fractions. The labelling pattern was slightly shifted on drug treatments from the 45*S*–65*S* to the 35*S*–45*S* region during 20 min pulse. This shift was more pronounced after a 90 min pulse and especially in the case of DAD treatment. An enhanced rate of processing was calculated from the data of the 90 min labelling experiments. Table 1 shows that the processing from 45*S* RNA to 28*S* RNA was acccelerated (cf. ratios of specific radioactivities of 28*S* and 45*S* RNAs) by a factor of 1.5–2 on DBD treatment especially for 18 h and by a factor of 2.5–3 on DAD treatment.

Further on, the synthesis of various types of RNAs was examined to reveal possible differences depending on the particular drug used. Synthesis of rRNA

was studied in nucleoli isolated from Novikoff hepatoma ascites tumor cells because the high endogenous RNase activity of Yoshida ascites made it impossible to obtain intact high molecular weight RNA from the nucleoli of these latter.

Table 1 shows that on treatment with either compound the specific radioactivity of whole noRNA increased by 20 to 30% above that of the control. The sucrose density gradient patterns of noRNA show (Fig. 1) that the noRNA is composed of 4–7*S*, 28*S*, 35*S*, 45*S*, 65*S* RNAs and some heavier species (Hidvégi et al., 1971). After the 20 min pulse labelling the radioactivity appeared in the 45*S*–65*S* RNA fractions which are precursors to 28*S* and 18*S* rRNAs (Hidvégi et al., 1971).

None of these drugs caused any change in the UV absorption patterns but the incorporation of ^{3}H-uridine into high molecular weight RNAs increased by 50 to 80% after 18 h treatment. This result indicates that DBD and DAD enhanced rRNA synthesis.

In the same experiment nRNA was also isolated and fractionated on poly(U)-Sepharose 4B column to separate poly(A)$^+$ RNA from poly(A)$^-$ RNA (Table 2).

Table 2

Assay of poly(A)$^+$ nRNA

The isolated nRNA of Novikoff hepatoma ascites tumor cells was labelled for 20 min intraperitoneally and separated on poly(U)-Sepharose 4B column as described in Methods. The counts in both fractions were added up. The ratio of the counts of the two fractions is the average of 3 to 4 experiments, standard deviation is given

Drug treatment	cpm of poly(A)$^+$ nRNA / cpm of poly(A)$^-$ nRNA
None	2.7 ± 0.3
DBD, 3 h	2.7 ± 0.3
DBD, 18 h	2.9 ± 0.3
DAD, 3 h	2.8 ± 0.3
DAD, 18 h	2.7 ± 0.3

Since the ratio of these two types of RNA did not change on drug treatment it may be assumed that the synthesis of mRNA also increased to a similar extent as that of rRNA.

The synthesis of LMW nRNA species was studied in Yoshida ascites tumor cells labelled for 90 min to get sufficient counts in these slowly synthesizing RNA species. The 4–10*S* RNA fractions from the sucrose density gradient were pooled, precipitated and further fractionated by polyacrylamide gel electrophoresis (Fig. 2). It can be seen that the untreated control contained 4*S* RNA, chromatin type 4.5*S* RNA, a mixture of 5*S* RNAs, 5.8*S* RNA as a shoulder of the U1 RNA and the uridylic acid rich RNA groups: U1, U2 and U3 RNAs (Nakamura et al.,

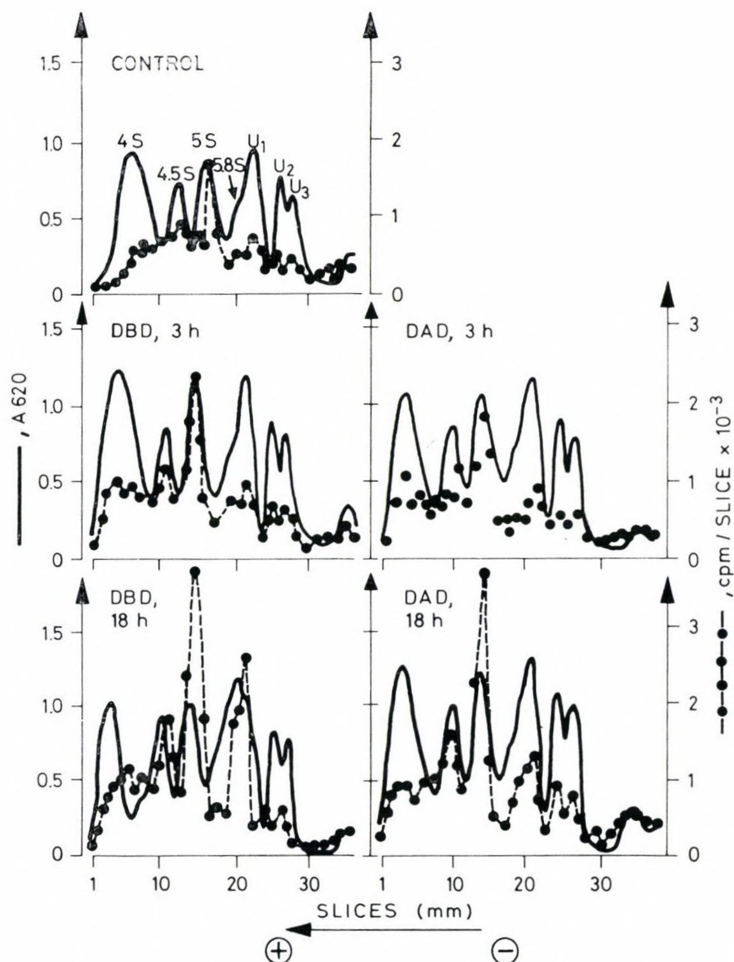

Fig. 2. Incorporation of ^{3}H-uridine into LMW nRNA. From sucrose gradients of nRNA labelled with ^{3}H-uridine for 90 min, as described in the legend of Fig. 1, fractions containing LMW nRNAs were pooled and RNA precipitated. The RNAs were fractionated on 10% (w/v) polyacrylamide gel for 12 h, 4 mA/tube at 4°C in 36 mM Tris/HCl and 30 mM phosphate buffer, pH 7.6, containing 1 mM EDTA. The gels were stained with 0.2% Methylene Blue in 0.4 M acetate buffer, pH 4.7, and scanned in a Chromoscan densitometer, cut into 1 mm slices (Mickle gel slicer) and the radioactivity was measured. The solid line represents the Chromoscan tracing at 620 nm and the dotted line the radioactivity. Direction of migration is indicated in the Figure

1968; Weinberg, Penman, 1968; Prestayko, Busch, 1968). In the control most of the counts were found in 4.5*S* RNA, 5*S* RNA and some in the uridylic acid rich RNA fractions. After 18 h drug treatment the label increased especially in 4.5*S*, 5*S* and U1 RNAs.

Binding of ^{3}H-DBD and ^{3}H-DAD to nRNA fractions

^{3}H-DAD treatment of Novikoff hepatoma cells *in vivo* for 3 h gave rise to 3 distinct radioactive peaks in nRNA fractionated by sucrose density gradient centrifugation with 20–23*S*, 45–50*S* and 90–100*S*, respectively. A substantial count was seen at the top of the gradient (Fig. 3) as well. The very same three peaks were present after 6 h treatment and a fourth peak also appeared at the top of the gradient. This latter was probably present after 3 h treatment as well, but it was masked by soluble radioactive metabolic products. After 18 h labelling, the first and fourth peaks diminished and labelled fractions appeared between these peaks. Using ^{3}H-DAD *in vitro* for 6 h gave a similar labelling pattern as that obtained after labelling *in vivo* for the same period of time, but the peaks were less distinct (Fig. 3).

Discussion

The data presented in this paper show that on DBD and DAD treatment the synthesis of high molecular weight nRNA increased. This applies to rRNA as well, since the experiments were performed also with RNA isolated from the nucleolus. Parallel to an increased synthesis, an enhanced processing of rRNA precursors was also observed.

The labelled nRNA was fractionated into RNAs containing either poly(A)$^{+}$ or poly(A)$^{-}$ groups at the 3' end. The fractions bound to poly(U)-Sepharose contain most of the pre-mRNA while the eluted fraction contains mainly pre-rRNA. Since drug treatment did not influence the ratio of these two fractions (Table 2) we might conclude that on drug treatment the synthesis of mRNA was enhanced to a similar extent as that of rRNA. The rate of poly(A) addition, however, could have been altered on DBD or DAD treatment as it has been reported for cordyceping treatment (Penman et al., 1970; Mendecki et al., 1972; Adesnik at al., 1972; Schumm, Webb, 1974).

Comparing the action of DBD and DAD on the synthesis of high molecular weight RNAs with that of other alkylating agents, striking differences can be seen. rRNA synthesis was inhibited by nitrogen mustard and CCNU (Abelson et al., 1974), it was not changed by a short exposure to BCNU (Rutman, Avadhani, 1976), while it was enhanced by DBD and DAD. mRNA synthesis was inhibited by nitrogen mustard, CCNU and BCNU, increased by cyclophosphamide (Rutman, Avadhani, 1976) and according to our preliminary assay probably also by DBD and DAD.

Apparently the length of the rRNA chains, synthetized in the presence of DBD or DAD, did not change since the UV absorption profile of the sucrose density gradient did not alter after treatment, as opposed to that obtained after treatment with nitrogen mustard (Rutman, Avadhani, 1976). Consequently, the premature termination of chain elongation after treatment with these agents can be ruled out.

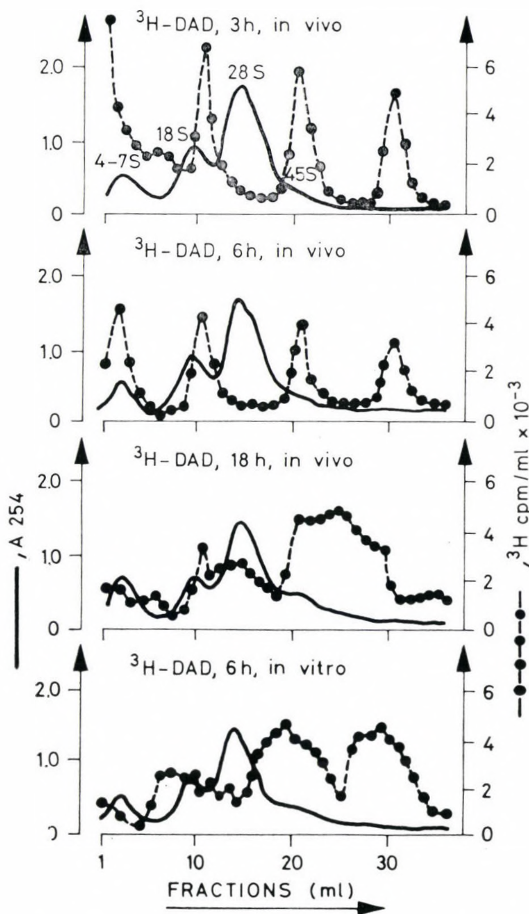

Fig. 3. Finding of ^{3}H-DAD to nRNA. In the *in vivo* labelling experiments 0.5 mCi (18.5 MBq)/1 mg) of ^{3}H-DAD or 0.5 mCi (18.5 MBq) /1 mg/ of ^{3}H-DBD was given i. p. to rats bearing Novikoff hepatoma ascites tumor cells which were then harvested after different times as indicated in the Figure. In the *in vitro* experiments 10^7 Novikoff hepatoma ascites tumor cells were incubated at 37 °Cin Parker 199 medium with 10% (v/v) bovine serum and shaken in a gyrotory shaker in an atmosphere of 95% air and 5% CO_2. One mCi (37 MBq) /2 mg/ ^{3}H-DAD was given to 100 ml cell suspension and the cells were collected 6 h later and washed several times. nRNA was then isolated and analyzed on a 10—45% (w/w) linear sucrose density gradient by centrifugation at 26 000 r. p. m. for 12 h

This is the first report on the behaviour of LMW nRNAs after treatment of cells with alkylating agents. Eighteen h treatment with DBD or DAD enhanced the labelling of 5*S* RNA and one of the uridylic acid rich RNA components (U1). A slight increase was observed with the 5.8*S* RNA component as well. There was an accumulation of uridylic acid rich RNA fractions on 18 h treatment. The role

of uridylic acid rich RNA components has not been revealed yet, but the exclusive localization of U3 RNA in the nucleolus (Prestayko et al., 1971) suggests its participation in rRNA synthesis.

Toyocamycin, known to block 45*S* RNA synthesis in the nucleolus, also blocks selectively the synthesis of 5.8*S* RNA and U3 RNA species (Hamelin et al., 1973). On the other hand, thioacetamide treatment enhanced the synthesis of both rRNA and uridine rich components of LMW nRNA (Ro-Choi et al., 1976). It was especially the U3 RNA and 5.8*S* RNA (and 4.8*S* RNA) species that accumulated (Ro-Choi et al., 1976). It is known that the 5.8*S* RNA is a processing product of 45*S* in the nucleolus (Maden, Robertson, 1974; Nazar et al., 1975). The involvement of 5.8*S* RNA and U3 RNA in rRNA synthesis suggests that the enhanced synthesis of 5*S*, 5.8*S* and uridylic acid rich RNA species upon treatment of the cells with DBD and DAD reflects an increased rRNA production of the cell.

The enhanced RNA synthesis can be correlated with the findings that the activity of both types of polymerase was enhanced in Yoshida ascites nuclei on DBD treatment (Institóris, Holczinger, 1976). Contrary to the suggestion that most of the clinically useful anticancer drugs block RNA synthesis (Abelson et al., 1974; Kann et al., 1974), enhancement of RNA synthesis by alkylating agents has also been reported (Wheeler, Bowdon, 1965; Peterson, Fox, 1970).

Binding experiments showed that the radioactivity derived from DBD or DAD was associated with four RNA fractions (Fig. 3). Since the RNA was subjected to hot phenol treatment, to several precipitations and separated by density gradient centrifugation, the appearance of label in definite fractions suggests a close association of (some derivative of) the drug with RNA, probably by covalent linkage. Interstrand crosslinks in RNA can be produced by bifunctional alkylating agents (Lawley et al., 1969; Shooter et al., 1971). We think that the hair pin loops of pre-mRNA seem to be a reasonable site of intramolecular crosslinks. The approximate sedimentation coefficients of our labelled RNA fractions (Fig. 3) were similar to those characteristic of the pre-mRNA classes (Perry, 1976) formed during processing after the elimination of intron sequences.

Antitumor alkylating agents are generally believed to share a common or similar ability to react with various intracellular nucleophilic sites resulting in structural and functional alterations of biopolymers that finally lead to cell death. It is also believed that among the biopolymers DNA represents the major target, i.e. inhibition of cell proliferation is explained as the consequence of DNA damage (Brookes, Lawley, 1961; Lawley, 1966). No special attention has been paid so far to the effect of alkylating agents on the RNAs in the cell and on RNA metabolism in general.

It should be emphasized that the action of cytostatic hexitols on RNAs does not exclude their damaging effect on DNA. We only would like to point out that relatively high doses of these drugs do not seem to affect drastically DNA function in terms of gene transcription: Neither reduction of the rate of RNA synthesis nor shortened RNA chains were observed in the nucleoli of cells treated with the cytostatic hexitols, DAD and DBD.

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Regulation by Phosphorylase Kinase of Phosphoprotein Phosphatase Activity: Simultaneous Control of Protein Phosphorylation and Dephosphorylation in Skeletal Muscle

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Phosphorylase kinase from rabbit skeletal muscle inhibited the dephosphorylation of phosphorylase *a* by phosphoprotein phosphatase. Phosphorylation (activation) of phosphorylase kinase by cyclic AMP-dependent protein kinase greatly increased this inhibitory effect. Thus, phosphoprotein phosphatase is inhibited by phosphorylase kinase in a reversible manner (Gergely et al. (1976) Biochim. Biophys. Acta *429* 809—816). In this paper the regulation by phosphorylase kinase at phosphoprotein phosphatase activity in different fractions of muscle extract and in the presence of various ligands has been investigated.

The presence of phosphorylase kinase also affected the ligand control of phosphatase activity. Phosphorylase kinase almost cancelled the inhibitory effect of AMP but hardly influenced the activating effect of glucose, glucose 6-phosphate and caffeine. Calmodulin, glycogen and phosphorylase *b* (effectors of phosphorylase kinase) did not influence the inhibitory effect of phosphorylase kinase.

Fractions of muscle extract also demonstrated the regulatory role of phosphorylase kinase. These fractions contained considerable amounts of phosphorylase kinase and phosphatase. Phosphatase activity was inhibited by phosphorylation reactions triggered by Mg^{++} and ATP. Heat-stable inhibitors were absent from these fractions, therefore the transient inhibition of phosphatase could be attributed to the phosphorylation of endogenous phosphorylase kinase. The introduction between phosphorylase kinase and phosphatase resulted in a loss of AMP sensitivity, i. e. AMP did not inhibit the activity of phosphatase in those fractions.

Our results imply that the phosphorylation of phosphorylase kinase is equally important both in the formation of enzymatically active phosphorylase *a* and in the inhibition of dephosphorylation of phosphorylase *a*. The consequence of these two effects is the elevated level of phosphorylase *a*.

Introduction

Interconversion of key enzymes between covalently modified and unmodified forms is an important mechanism in cellular regulation. Glycogen metabolism of skeletal muscle was the first example of covalent modification by reversible phosphorylation and dephosphorylation processes. It is known that the degradation and synthesis of glycogen is regulated by the interconversion of the two forms

Abbreviations: Cyclic AMP, adenosine 3' : 5' monophosphate; ATP-γ-S, adenosine 5'-0-(3-thiotriphosphate); Glc-6-P, glucose 6-phosphate.

of phosphorylase, phosphorylase kinase and glycogen synthase (reviewed by Krebs, Beavo, 1979; Chock et al., 1980; Cohen, 1978). For the phosphorylation and dephosphorylation of enzymes to serve a regulatory function, these processes must be regulated. This in turn requires that the protein kinases and phosphatases be controlled. The control of kinases is well established by specific effector compounds or messengers. The phosphoprotein phosphatases (phosphatases) are also subject to different regulatory effects. Ligands and proteins can influence the dephosphorylation of muscle or liver phosphorylase *a*. In some cases the mechanism of control is known (Martensen et al., 1973a, 1973b; Hers, 1976; Bot et al., 1977; Gergely et al., 1976; Gergely, Bot, 1977; Nimmo, Cohen, 1978b).

The most effective regulation of glycogen metabolism should be attained by a co-ordinated control of the interconverting kinases and phosphatases. Experiments with a protein-glycogen complex isolated from rabbit skeletal muscle demonstrated that activation of phosphorylase takes place synchronously with the inhibition of phosphatase during the phosphorylation processes (Fischer et al., 1971). The transient inhibition of phosphatase also occurs in muscle or liver extracts during the phosphorylation processes (Gergely et al. 1978; Dombrádi et al. 1978). The inhibition of phosphatase could not be explained by the effect of ligands (Fischer et al. 1971; Haschke et al. 1972) but is attributed to the presence of proteins (Gergely et al., 1976, 1978). Thus protein, protein interactions are involved in the co-ordinated regulation of phosphatase. One possible candidate for the control of phosphatase is phosphorylase kinase. It has been demonstrated that phosphorylase kinase is phosphorylated in suspensions of a protein–glycogen complex a potent inhibitor of phosphatase (Yeaman, Cohen, 1975; Gergely et al., 1976). The inhibition is reversible since it greatly decreases with the dephosphorylation of kinase (Gergely et al., 1976; Gergely, Bot, 1978). The regulatory subunit of cyclic AMP-dependent protein kinase also inhibits the dephosphorylation of phosphorylase *a* and this inhibition is due to its interaction with phosphorylase rather than with phosphatase (Gergely, Bot, 1977). Heat-stable inhibitors may inhibit the activity of phosphatase, too. In the case of inhibitor-1 the inhibitory effect is reversible (Nimmo, Cohen, 1978a) and the phosphorylation of inhibitor-1 also occurs *in vivo* (Tóth et al., 1977, Foulkes, Cohen, 1979). Thus, the transient inhibition of phosphatase is effected by phosphorylated phosphorylase kinase, the regulatory subunit of protein kinase and heat-stable inhibitor(s).

In the past years several phosphatase preparations have been made that differed in substrate specificity and molecular weight. The reports of Laloux et al. (1978) and Laloux and Hers (1979) that phosphatase activity is not inhibited by inhibitor-1 in freshly prepared muscle and liver extracts, suggest that the effect of inhibitory proteins might be diverse for different phosphatase preparations.

The present work was undertaken to investigate the regulatory role of phosphorylase kinase in the control of the inhibition of phosphatase. It will be shown that the transient inhibition of phosphatase occurs in the isoelectric precipitate and ammonium sulfate fractions of rabbit skeletal muscle extract and that

this inhibition is not due to the presence of heat-stable inhibitors. We paid a particular attention to the problem of phosphatase preparations, too. In most experiments no exogenous phosphatase was added, therefore it is the properties and regulation of endogenous phosphatase that have been investigated.

Materials and methods

Materials

Glucose-6-phosphate and trypsin were obtained from Calbiochem; cyclic AMP, ATP and ATP-γ-S from Boehringer; ammonium sulfate, EGTA, glucose, mercaptoethanol, Mg acetate, NaF and Na-β-glycerophosphate from Merck; Sephadex G-200 and Blue Dextran 2000 from Pharmacia; EDTA and Norit A from Serva; caffeine, bovine serum albumin, soybean trypsin inhibitor and Tris from Sigma; [γ-^{32}P]ATP from the Hungarian Isotope Institute.

Glycogen was prepared from rabbit liver (McCready, Hassid, 1955) and glucose 1-phosphate from potato (Helmreich, Cori, 1964), both were treated with Norit A.

Enzyme preparations

All enzymes were isolated from rabbit skeletal muscle. Phosphorylase *b* (Fischer, Krebs, 1958) and non-activated phosphorylase kinase (Cohen, 1973) were purified to homogeneity. Phosphoprotein phosphatase was purified according to the method of Brandt et al. (1975). ^{32}P-labelled phosphorylase *a* was prepared from phosphorylase *b* using purified phosphorylase kinase and [γ-^{32}P] ATP (Bot et al., 1975). Thiophosphate-activated and phosphorylated phosphorylase kinases were prepared as described previously (Gergely et al., 1976). Phosphorylase kinase and phosphatase preparations were stored at $-25°$ in 40 mM Tris/10 mM mercaptoethanol, 2 mM EDTA, pH 7.0, and 50% glycerol. Prior to use glycerol was removed by dialysis against 100 vol of 40 mM Tris/10 mM mercaptoethanol, pH 7.4, at 4°.

Calmodulin was prepared from bovine brain according to the method of Watterson et al. (1976).

Assay of enzymes

Enzyme analysis was carried out at 30°. Phosphorylase was assayed as described previously (Bot et al., 1977). Phosphorylase kinase activity was measured by the method of Cohen (1973). The concentration of protein was measured according to Lowry et al. (1951) with bovine as a standard.

Phosphatase activity was assayed by ^{32}P-labelled phosphorylase *a* as a substrate. Two hundred microliters of the phosphatase sample was incubated with 50 μl (0.2 mg) of phosphorylase *a* in 40 mM Tris/10 mM mercaptoethanol, pH 7.4, in the presence of 5 mM caffeine. Aliquots were withdrawn after 20, 50, 90 and 120 sec, and immediately mixed with 100 μl of bovine serum albumin (20 mg/ml).

The reaction was stopped by the addition of 1 ml of 10% TCA. The samples were centrifuged (2000 × *g*, 5 min) and aliquots were removed from the supernatants. The radioactivity was measured in a scintillation spectrometer in 5 ml 0.25 N NaOH using the Cerenkov effect of ^{32}P. One unit of phosphatase activity is defined as the amount of enzyme which converts 1 nmol of dimeric phosphorylase *a* to phosphorylase *b* per min (Brandt et al., 1975).

Preparation of muscle extract and fractionation

Normally fed New Zealand white rabbits (2.5–3 kg body weight) were used in all experiments. 300 mg sodium pentabarbiturate was injected to the marginal ear vein of the animal. After 10 min a lethal dose of sodium pentabarbiturate was given and the rabbit was exsanguinated, skinned and the muscle from the hind limbs and back was removed and placed into 2 vol of ice cold 4 mM EDTA/4 mM EGTA, pH 8.0. The muscle (about 400 g) was minced and homogenized in a Waring blendor at 0°. The pH was maintained at 8.0 by addition of 2 M Tris. The entire operation was completed within 5 min. All subsquent steps were carried out at 0–4°.

The homogenate was centrifuged at 12 000 × *g* for 10 min and the supernatant fluid decanted through glass wool. The pH was adjusted to 6.1 by 1 M acetic and kept at this value. After standing for 30 min the isoelectric precipitate was collected by centrifugation (12 000 × *g*, 10 min). In some experiments further isoelectric precipitates were removed at pH 5.7 or 5.1 from the clear supernatant. The pH 6.1 precipitate was suspended in 40 mM Tris/10 mM mercaptoethanol, pH 7.4, to give a protein concentration of 40–50 mg/ml.

The suspension of the pH 6.1 precipitate was then centrifuged (78 000 × *g*, 60 min) and the supernatant fluid was fractionated by ammonium sulphate. Two fractions (0–35% and 35–60%) were made by adding solid ammonium sulphate. During this operation, the pH was maintained at 7.4. The precipitates were collected by centrifugation, dissolved and dialyzed against 40 mM Tris/10 mM mercaptoethanol, pH 7.4. The protein concentration of the dialysed fractions was in the range of 30–35 mg/ml.

Transient and permanent inhibition of phosphatase

A suspension of an isoelectric precipitate or an ammonium sulfate fraction was incubated at 30° in the presence of 3 mM Mg acetate and 5 μM cyclic AMP (final protein concentration 20–25 mg/ml). The phosphorylation reaction was initiated by addition of 0.45 mM ATP or 0.45 mM ATP-γ-S. In the case of ATP the inhibition of phosphatase was transient while a permanent inhibition was observed with ATP-γ-S. Two hundred microliter aliquots were withdrawn from the incubation mixture and assayed immediately for phosphatase activity as described above. Initial velocity was determined by the liberation of ^{32}P from the ^{32}P-labelled phosphorylase *a*. Phosphatase activity in the absence of Mg-ATP was taken as 100%.

Treatment with trypsin

This treatment was applied to the samples of different phosphatases inhibited either by phosphorylation processes (with ATP) or thiophosphorylation reactions (with ATP-γ-S). The samples (200 μl) were incubated for 10 min at 30° with 10 μl of trypsin solution (2 mg/ml). The trypsinization was stopped by the addition of 10 μl of soybean trypsin inhibitor (10 mg/ml, 5-fold excess by weight). After the trypsin treatment the phosphatase activity was assayed as above.

Purified phosphorylase kinase was also treated with trypsin. Phosphorylase kinase (2.4 mg) was mixed with 5 μg trypsin in 40 mM Tris/10 mM mercapto-ethanol, pH 7.4, at 30°. Aliquots were withdrawn and the reaction stopped by the addition of soybean trypsin inhibitor. Then the activity of phosphorylase kinase was assayed at pH 6.8 and 8.2. The inhibitory effect on the dephosphorylation of phosphorylase *a* of trypsin-treated phosphorylase kinase was also checked.

Isolation of inhibitor-1

Inhibitor-1 was isolated during the different steps of the purification procedure both before and after activation with 3 mM Mg acetate, 0.3 mM ATP-γ-S, 5 μM cyclic AMP and protein kinase by the method of Foulkes and Cohen (1979) with the exception of the chromatography on Sephadex G-100.

The assay of inhibitor-1 was carried out as described by Nimmo and Cohen (1978a) using purified phosphatase. The activity of inhibitor-1 is expressed as the percentage of the inhibitory capacity of non-phosphorylated starting material.

Results

Inhibition of phosphoprotein phosphatase with purified phosphorylase kinase

We have reported previously that phosphorylase kinase inhibits the dephosphorylation of phosphorylase *a* by phosphatase. Figure 1 shows that the release of ^{32}P from ^{32}P-labelled phosphorylase *a* was inhibited by phosphorylase kinase. It also shows that the phosphorylated (activated) form of kinase was more inhibitory than the non-activated one. A delay in the dephosphorylation of phosphorylase *a* was also observed in the presence of phosphorylated kinase. The dephosphorylation of phosphorylase *a* could start only after the dephosphorylation of kinase, confirming our earlier results (Gergely et al., 1976).

The thiophosphate-activated form of phosphorylase kinase was also inhibitory to the phosphatase reaction [documented in our previous work (Gergely et al., 1976)]. This new form of kinase is resistant to the action of phosphatase and behaves as a strong inhibitor simulating the effect of phosphorylated kinase.

Effectors modifying the inhibition caused by phosphorylase kinase

Ligand sensitivity. In the next series of experiments the inhibitory action of phosphorylase kinase was tested in the presence of various effectors (Fig. 2). The thiophosphate-activated form of kinase was applied which resulted in about 50

per cent decrease of the activity of phosphatase. Phosphorylase *b*, a substrate of phosphorylase kinase, and glycogen can bind to kinase as reported in the literature (Krebs et al., 1964; Bot et al., 1971; Carlson, Bechtel, 1979). Neither glycogen nor phosphorylase *b* had any influence on this inhibition. Moreover, a combination of the two did not alter the activity of phosphatase. It is known that phosphorylase kinase has an absolute Ca^{++} requirement for its catalytic function and calmodulin plays an essential role in manifesting the effect of Ca^{++}. Addition of extra calmodulin to phosphorylase kinase preparations increased the catalytic activity of kinase, too (Cohen, 1978; Shenolikar et al., 1979). As shown in Fig. 2, Ca^{++} had no effect on the inhibitory action of phosphorylase kinase and addition of calmodulin did not influence the inhibition.

Various ligands are known to affect the phosphatase reaction by modifying the substrate phosphorylase *a*. The best known examples are AMP, glucose and caffeine (Bot, Dósa, 1971; Martensen et al., 1973a, b; Bot et al., 1977). Inorganic phosphate inhibits the phosphatase reaction by binding to phosphatase as deduced from the investigations of Martensen et al. (1973a). The question arises whether or not the action of phosphorylase kinase in the phosphatase reaction is altered by modified proteins. As shown in Table 1, the nucleotide AMP was less inhibitory to the phosphatase reaction in the presence of thiophosphate-activated kinase. The presence of kinase almost blocked the inhibitory effect of AMP. The inhibitory

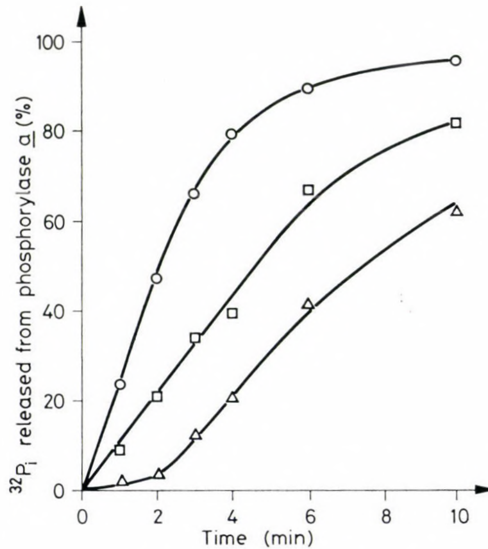

Fig. 1. Effect of phosphorylase kinase on the dephosphorylation of phosphorylase *a*. The dephosphorylation mixture contained ^{32}P-labelled phosphorylase *a*, purified phosphatase and phosphorylase kinase. *Additions:* none (○), 0.12 mg/ml non-activated phosphorylase kinase (□), 0.12 mg/ml activated phosphorylase kinase (△)

Table 1

Effect of thiophosphate-activated phosphorylase kinase on the ligand sensitivity of purified phosphatase

Phosphatase activity was determined as described in Materials and methods. The ligands were dissolved in 40 mM Tris/10 mM mercaptoethanol, pH 7.4, and added to the incubation medium at the indicated concentrations. The dephosphorylation of phosphorylase *a* was measured in the absence and presence of 0.03 mg/ml thiophosphate-activated kinase. Phosphatase activity is expressed as the percentage of the initial reaction rate measured with or without kinase. The absolute value of the reaction rate was 255 pmole $^{32}P_i$ released/min without kinase and 140 pmole $^{32}P_i$ released/min with thiophosphate-activated kinase

Ligands	Phosphatase activity (%)	
	+ kinase	− kinase
none	100	100
AMP 5 μM	41	95
AMP 20 μM	6	81
P_i 5 mM	47	53
Glucose 20 mM	138	117
Caffeine 1 mM	123	115

effect of inorganic phosphate was not altered by the addition of kinase. The activating effect of glucose and caffeine also appeared in the presence of kinase, although it was reduced to some extent.

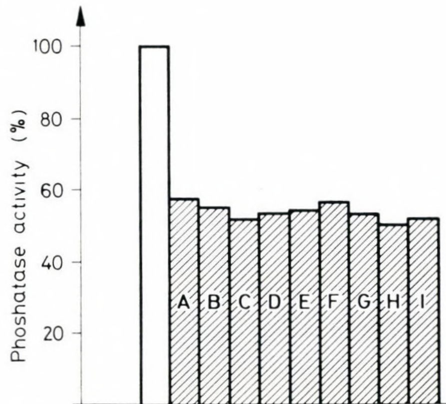

Fig. 2. Influence of effectors on the inhibition of phosphatase caused by thiophosphate-activated phosphorylase kinase. For details see Materials and methods. The ordinate shows the activity of phosphatase in per cent. Open column: activity of phosphatase in the absence of kinase; dashed columns: activity of phosphatase in the presence of 0.03 mg/ml thiophosphate-activated phosphorylase kinase. *Additions:* none (A), 0.8% glycogen (B), 2.3 mg/ml phosphorylase *b* (C), 0.8% glycogen + 2.3 mg/ml phosphorylase (D), 2.7 mM EGTA (E), 10 μM Ca^{++} (F), 100 μM Ca^{++} (G), 100 μM Ca^{++} + 2.4 μM calmodulin (H), 100 μM+Ca^{++} + 24 μM calmodulin (I)

Trypsin treatment. The trypsin sensitivity of the inhibition caused by phosphorylase kinase was also studied. The effect of trypsin was monitored by determination of the specific activity of kinase at pH 6.8. It is seen from Fig. 3 that trypsinization resulted in an increase in the specific activity of both forms of kinase. This is in good agreement with earlier findings (Cohen, 1973). The amount of trypsin was chosen so as to give a maximum effect on the increase of specific activity after 5 min. Trypsinization also influenced the inhibitory effect of kinase.

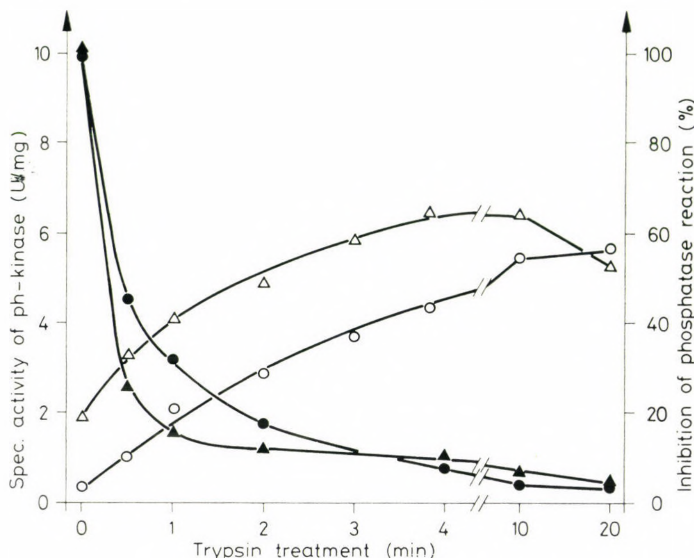

Fig. 3. Influence of trypsin treatment on the inhibitory effect and specific activity of phosphorylase kinase. Non-activated phosphorylase kinase (○, ●) and thiophosphate-activated phosphorylase kinase (△, ▲) were treated with trypsin as described in Materials and methods. Increase in specific activity of phosphorylase kinase at pH 6.8 (○, △) and decrease in the inhibitory effect of phosphorylase kinase samples (●, ▲) during trypsin treatment. The inhibitory effect of untreated kinase samples in the phosphates reaction was taken as 100 per cent

A short period (1 min) of trypsin treatment greatly diminished the inhibition of phosphatase reaction by phosphorylase kinase. Further trypsin treatment of kinase fully abolished its inhibitory effect.

Inhibition of phosphatase in pH 6.1 isoelectric precipitate

The pH 6.1 precipitate obtained from rabbit skeletal muscle extract contained phosphorylase kinase and a considerable amount of phosphatase (see Table 3). Ax shown in Fig. 4 the addition of cyclic AMP and Mg-ATP triggered an immediate inhibition of phosphatase. Phosphatase became transiently inhibited as tested by exogenous substrate, ^{32}P-labelled phosphorylase *a*. After maximum,

reversible inhibition the phosphatase activity started to increase and after 15 min resumed its initial value. Upon addition of ATP-γ-S a *permanent* inhibition of phosphatase was observed and only trypsinization of the samples cancelled the inhibition. The use of ATP-γ-S is based on the demonstration that ATP-γ-S serves as a substrate for several protein kinases, but the thiophosphorylated proteins are not substrates for phosphatase (Gratecos, Fischer, 1974; Gergely et al., 1976; Shery et al., 1978), thus the protein is trapped in the phosphorylated state. Therefore the formation of thiophosphorylated protein(s) caused a permanent inhibition of phosphatase. This inhibition was sensitive to proteolytic attack

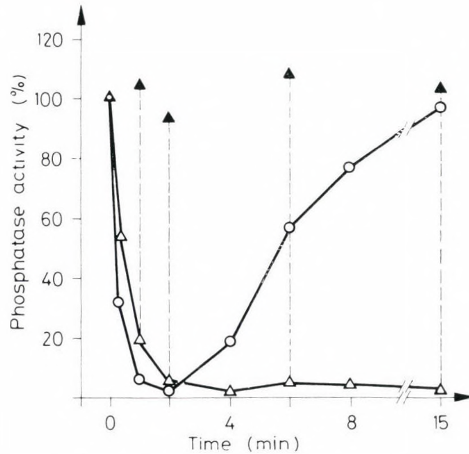

Fig. 4. Transient and permanent inhibition of phosphatase in a pH 6.1 precipitate. Conditions of the activation process and phosphatase assay are described in Materials and methods. Decrease of phosphatase activity upon addition of ATP (○) and ATP-γ-S (△). Phosphatase activity in the absence of ATP or ATP-γ-S is taken as 100%. Phosphatase activity of ATP-γ-S-treated samples after trypsinization (▲). Trypsin treatment increased about 2.5-fold the activity of phosphatase in the pH 6.1 precipitate. Therefore, the phosphatase activity of trypsin-treated samples is expressed as the percentage of this elevated value

since trypsin fully suppressed the inhibition of phosphatase. These results imply that the phosphorylation of some protein component(s) in the pH 6.1 precipitate may regulate the activity of phosphatase. There are several candidates for this role.

Inhibitor-1 is a heat-stable protein which inhibits phosphatase only in its phosphorylated form. Inhibitor-1 has been shown to be phosphorylated in skeletal muscle both *in vitro* (Nimmo, Cohen, 1978a) and *in vivo* (Tóth et al., 1977; Foulkes, Cohen, 1979; Foulkes et al., 1980). In spite of this fact inhibitor-1 cannot regulate the activity of phosphatase in the pH 6.1 precipitate since virtually all inhibitor-1 remained in the pH 6.1 supernatant (Table 2). The pH 6.1 precipitate was therefore considered as an inhibitor-free material since the presence of heat-stable inhibitor was not detected after thiophosphorylation either. A considerable amount of

Table 2

Distribution of heat-stable inhibitor-1 during the fractionation of muscle extract

The procedure for preparing heat-stable inhibitor-1, thiophosphorylation of muscle fraction and assay of inhibitor-1 are described] in Materials and methods

Step	Inhibitor-1 activity (%)	
	Before thio-phosphorylation[a]	After thio-phosphorylation[b]
Muscle extract (pH 7.4)	100	315
pH 7.4—6.1 precipitate	0	2
pH 6.1 supernatant	98	330
pH 6.1—5.7 precipitate	26	47
pH 5.7—5.1 precipitate	55	195

[a] Average of four determinations
[b] Average of two determinations

inhibitor-1 found in the pH 6.1 supernatant was precipitated between pH 75. and 5.1. The precipitates between pH 6.1 and 5.1 also contained phosphatase. This enabled us to carry out inhibition studies with Mg-ATP or Mg-ATP-γ-S similarly to the experiments done with the pH 6.1 precipitate. ATP caused only a 40% and reversible inhibition of the endogenous phosphatase in the pH 6.1 – 5.7 precipitate.ATP-γ-S did not increase the extent of phosphatase inhibition. Phosphatase activity of the pH 5.7 – 5.1 precipitate was completely blocked by either ATP or TP-γ-S, but upon addition of ATP a permanent inhibition was observed.

Properties of phosphatase in Fractions I and II

Two ammonium sulphate fractions (Fraction I and II) were isolated from the high speed supernatant of the pH 6.1 precipitate (Table 3). Preparation of Fraction I is similar to the purification of phosphorylase kinase as reported by Cohen (1973). During preparation the enzymatic activities of phosphorylase kinase and phosphatase were tested. It is seen from Table 3 that Fraction I can be considered as a "kinase rich" and Fraction II as a "phosphatase rich" part of the preparation.

Further evidence for the difference of phosphatase existing in Fractions I and II was obtained by activation and inhibition studies. It is known that trypsin treatment or ethanol precipitation can enhance the activity of phosphatase (Brandt et al., 1975; Laloux et al., 1978). Both treatment resulted in an increase of phosphatase activity though the extent varied in Fractions I and II (Table 4). The activity of phosphatase in these Fractions was also regulated by phosphorylation processes. A transient inhibition of phosphatase was observed upon addition of ATP and a permanent one with ATP-γ-S in Fraction I. Trypsin cancelled the

Table 3

Isolation of Fractions I and II from rabbit skeletal muscle

420 g of rabbit skeletal muscle was used in this preparation. Details of the procedure are described in Materials and methods

Step	Protein mg/ml	Phosphorylase kinase		Phosphorylase phosphatase	
		Total activity units	Spec. activity U/mg	Total activity units	Spec. activity U/mg
Extract (pH 7.4)	46	1650	0.06	7680	0.28
pH 6.1 precipitate	37.5	1080	0.18	2840	0.47
78 000 × *g* supernatant	23.5	620	0.53	965	0.82
Fraction I (0–35% am. sulfate prec.)	46.0	528	2.46	205	0.95
Fraction II (35–60% am. sulfate prec.)	54.5	46.2	0.096	1795	3.72

Table 4

Activation and inhibition of phosphatase activity in Fraction I and Fraction II

The treatment of the fractions with trypsin is described in Materials and methods. Ethanol activation of samples was performed according to Brandt et al. (30). Transient and permanent inhibition of phosphatase was carried out as described in the text. Phosphatase activity is expressed as the percentage of that of the non-treated sample

Treatment	Phosphatase activity (%)	
	Fraction I	Fraction II
none	100	100
+ trypsin	210	165
ethanol precipitation	430	760
+ 0.45 mM ATP (3 min incubation)	5	52
+ 0.45 mM ATP (25 min incubation)	92	79
+ 0.45 mM ATP- -S	9	57
0.45 mM ATP- -S + trypsin	178	153

inhibition caused by ATP-γ-S. Phosphatase in Fraction II was less inhibited by either ATP or ATP-γ-S.

Control of phosphatase with ligands in Fractions I and II

Since the dephosphorylation of phosphorylase *a* is also under a complex regulation brought about by ligands, in particular by nucleotides, sugars and sugar esters, the effect of various ligands on phosphatase activity was investigated, as summarized in Table 5. The data show that AMP was a much stronger inhibitor of phosphatase occurring in Fraction II than that in Fraction I. AMP caused a 50% inhibition at micromolar concentrations when Fraction II was tested, while 0.2 mM AMP was necessary for the 50% inhibition of phosphatase in Fraction I. Glucose, glucose 6-phosphate and caffeine increased the activity of phosphatase in both Fractions. They also acted on the inhibition of phosphatase by AMP. Caffeine was the most effective in cancelling the inhibition by AMP.

Table 5

Role of ligands in the regulation of phosphatase Fraction I and Fraction II

Phosphatase activities are determined as described in Materials and methods. Activities in the absence of ligands are taken as 100%

Additions	Phosphatase activity (%)	
	Fraction I	Fraction II
none	100	100
AMP 1 μM	100	78.1
5 μM	96.3	32.4
40 μM	73.4	1.0
40 μM (without preincubation)	62.8	0
Glucose 20 mM	111.5	103.2
Glc-6-P 5 mM	132.6	112.4
Caffeine 5 mM	172.8	155.3
AMP 5 μM		
+ glucose 20 mM	—	54.6
+ Glc-6-P 5 mM	—	79.8
+ caffeine 5 mM	—	114.9
AMP 40 μM		
+ glucose 20 mM	84.7	—
+ Glc-6-P 5 mM	98.6	—
+ caffeine 5 mM	146.5	—

The Fractions were usually preincubated for 2 min with ligands and addition of phosphorylase *2* initiated the phosphatase assay, which was completed in 2 min (see Methods). Since the Fractions should contain AMP deaminase activity, the experiment was repeated without preincubation and the assay was completed in 30 sec. According to Table 5 the inhibition caused by AMP was essentially the same with or without preincubation.

Discussion

The results presented in this paper show that phosphorylase kinase plays an important role in the regulation of phosphatase. This confirms our previous findings based on experiments performed with purified enzymes. Phosphorylase kinase inhibits the dephosphorylation of phosphorylase *a* catalyzed by phosphatase. In this respect the phosphorylated form of kinase is particularly important: it even causes a delay in the dephosphorylation of phosphorylase *a*. The finding that phosphorylated (activated) phosphorylase kinase is a stronger inhibitor than the non-activated one explains the reversible inhibition of phosphatase (Gergely et al., 1976; Gergely, Bot, 1978).

It is known that the enzymatic activity of kinase is influenced by the presence of Ca^{++} and calmodulin (Cohen, 1978; Shenolikar et al., 1979). Furthermore, phosphorylase *b* and glycogen can bind to kinase preparations (Carlson et al., 1979). Despite these facts neither of the modifiers altered the inhibitory effect of phosphorylase kinase in the phosphatase reaction. It seems that the inhibitory action of kinase is not connected with its catalytic function or its ability of binding macromolecules. The inhibitory action of kinase was tested in the dephosphorylation reaction of phosphorylase *a*. It is well established that various ligands can influence this reaction by modifying the conformation of phosphorylase *a*. Thus the reduced inhibitory effect of AMP in the presence of kinase (Table 1) could be attributed to an interaction between phosphorylase *a* and kinase. This in turn suggests that AMP binding sites of phosphorylase *a* are less accessible or that their affinity decreases under the effect of kinase. The inhibition caused by inorganic phosphate was the same in the absence and presence of kinase. This observation may be interpreted in two ways. Firstly, the binding of kinase does not modify the phosphate binding sites of phosphorylase *a*. Secondly, phosphate inhibits phosphatase rather than phosphorylase *a* which was originally suggested by Martensen et al. (1973a). The latter explanation does not exclude an interaction between phosphatase and kinase.

It seems that the inhibitory effect of phosphorylase kinase is extremely sensitive to proteolytic attack. The region which is responsible for the inhibition could be located on an exposed site of one of the four dissimilar subunits in kinase. Since phosphorylation of the beta subunit increases the inhibitory effect of phosphorylase kinase, it is supposed that the beta subunit plays the inhibitory role in the regulation of phosphatase.

The regulatory role of phosphorylase kinase was also demonstrated in the acid precipitate and ammonium sulfate fractions of muscle extract. We have shown previously that the phosphatase activity of muscle extracts is transiently inhibited by protein phosphorylation mediated by the action of cyclic AMP-dependent protein kinase (Gergely et al., 1978). Several proteins are phosphorylated under these conditions but two candidates (phosphorylase kinase and inhibitor-1) are the most likely since they can inhibit the phosphatase reaction and their inhibitory effect is greatly enhanced by phosphorylation reaction. The regulatory role of inhibitor-1, a heat-stable protein, has been demonstrated by Cohen and his associates. They also reported that the degree of phosphorylation of inhibitor-1 is influenced *in vivo* in response to adrenaline (Foulkes, Cohen, 1979) or insulin (Foulkes et al., 1980). The physiological role of inhibitor-1 (and other heat-stable inhibitors) appears doubtful since they do not inhibit the activity of "native" phosphatase (Laloux et al., 1978; Laloux, Hers, 1979).

Our experiments carried out with the suspension of the pH 6.1 isoelectric precipitate confirm the physiological implication of phosphorylase kinase in the inhibition of phosphatase. The pH 6.1 precipitate did not contain inhibitor-1 (see Table 2) or other heat-stable inhibitor proteins. It was also demonstrated that inhibitor-2, a further protein inhibitor of phosphatase, was located in the supernatant fraction of pH 6.1 precipitate (Foulkes, Cohen, 1980). Accordingly, pH 6.1 precipitate or Fractions I and II could be considered as heat-stable inhibitor-free materials. Thus in the transient inhibition of phosphatase observed upon addition of Mg-ATP and cyclic AMP, phosphorylase kinase itself may be involved. Protein-protein interactions between phosphorylase kinase and phosphatase were detected by frontal gel filtration of muscle extract in this laboratory (Gergely et al., 1974). The complex also contained phosphorylase. Sticking of phosphatase to phosphorylase kinase can be deduced from the data of the purification procedure of kinase (Table 3). It was also demonstrated that highly purified phosphorylase kinase preparations did contain some phosphatase activity (unpublished observation). Accordingly, two phosphatase fractions were obtained upon ammonium sulfate precipitation. Phosphatase in Fraction I, which precipitated at 0–35% ammonium sulfate, was bound to phosphorylase kinase and Fraction II contained only a slight amount of kinase. The control of phosphatase activity was different in these Fractions. A remarkable difference was observed in the ligand sensitivity of the two fractions. Phosphatase activity of Fraction I was insensitive to inhibition caused by AMP. A similar observation, i.e. a lack at inhibition of phosphatase activity by AMP in suspensions of a protein-glycogen complex, has also been reported by Fischer et al. (1971) and Haschke et al. (1972). No clear explanation of this unusual behaviour of the complex has been offered. Several lines of evidence indicate that phosphorylase kinase is responsible for the insensitivity of phosphatase to AMP inhibition. The inhibitory effect of AMP greatly decreased when a phosphorylase kinase preparation was added to purified phosphatase (Table 1). AMP at micromolar concentrations did not inhibit the activity of phosphatase occurring in Fraction I and only high concentrations of AMP did cause a slight

inhibition (Table 5). It is concluded that the complex of phosphorylase kinase, phosphatase and phosphorylase *a* suppressess the regulatory function of AMP probably by preventing the conformational changes that result from the binding of ligands to the proteins.

Our data indicate the importance of phosphorylase kinase in the regulation of phosphatase. The transient and reversible inhibition of phosphatase during protein phosphorylation is well documented. The process is cyclic AMP dependent and mediated by a protein kinase. This leads to the formation of enzymatically active phosphorylase *a*, a process catalyzed by phosphorylated (activated) phosphorylase kinase. Phosphorylated kinase also regulates the dephosphorylation of phosphorylase *a* by inhibiting this reaction. Our results imply that the phosphorylation of phosphorylase kinase may be equally important both in the formation of phosphorylase *a* and in the inhibition of phosphatase. Both, in turn, result in an elevation of the level of phosphorylase *a*.

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Steroid Spectrum in Human Urine as Revealed by Gas Chromatography V.

Identification and Quantitation of 3α, 20α-dihydroxy-5-β pregnan-11-one (11-keto-pregnanediol) during Different Stages of Development in Children with C/21 Hydroxylase Deficiency

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A component was observed in the steroid spectrum of the urine of salt-loosing children with C/21-hydroxylase deficiency, which was eluted from Sp 2100 stationary phase before pregnanetriol but, unlike pregnanetriol, exhibited heat and acid stability. This component was isolated by paper chromatography and identified as 3α, 20α-dihydroxy-5β-pregnan-11-one by GC-MS and further gas chromatographic analysis. The amount of the steroid was minimal in the urine of infants, while in children submitted to substitution corticoid therapy it showed an increasing tendency, especially during puberty. The maximal value of excretion, in one case, amounted to 17% relative to total steroids. In puberty a significant excretion of 11-keto-pregnanediol indicates that under the given conditions the 11β-hydroxylation of steroid intermediates in the adrenals may be considerable not only at the level of 11-hydroxy-progesterone but also at that of progesterone.

Introduction

In our last publication on the investigation of the steroid excretion of children with C_{21}-hydroxylase deficiency (Kecskés et al., 1981) we reported the presence of some components the retention characteristics of which differed from those of steroids previously observed in the steroid profile of children with C_{21}-hydroxylase deficiency (Völlmin, 1971; Desgres et al., 1974; Shackleton, 1976).

Abbreviations: A, 3α-hydroxy-5α-androstan-17-one (androsterone), Chl, 5-cholesten-3β-01 (cholesterol); DHA, 3β-hydroxy-5-androsten-17-one; E, 3α-hydroxy-5β-androsten-17-one (etiocholanolone); FID, flame ionization detector; GC-MS, gas chromatography combined with mass spectrometry; I, Kováts' retention index; IST, internal standard; 11OA, 3α-hydroxy-5α-androstane-11,17-dione (11-keto-androsterone); 11-OE, 3α-hydroxy-5β-androstane-11,17-dione (11-keto-etiocholanolone); 11-OPd, 3α, 20α-dihydroxy-5β-pregnan-11-one (11-keto-pregnanediol); 11-OPt, 3α,17β,20α-trihydroxy-5β-pregnan-11-one (11-keto-pregnanetriol); 11-OHA, 3α,11β-dihydroxy-5α-androstan-17-one (11-hydroxyandrosterone), 11-OHE, 3α,11β-dihydroxy-5β-androstan-17-one (11-hydroxy-etiocholanolone); 16-OH-DHA, 3β16α/16β-dihydroxy-5-androsten-17-one (16-hydroxy-DHA); 17-OH Pg, 3α,17α-dihydroxy-5β-pregnan-20-one(17-hydroxy-pregnanolon); Pd, 5β-pregnane-3α, 20α-diol (pregnanediol); Δ5-Pd, 5-pregnane-3β, 20α-diol (pregnenediol); PI, 5β-pregnane-3α,17α, 20α-triol (pregnanetriol); Δ5 PT, 5-pregnene-3β,17α, 20α-triol (Δ5-pregnenetriol); RRT_{ch}, retention time relative to 5α-cholestane; Sp 2100, methyl-substituted polysiloxan, stationary phase; UR, unresolved component;

In our present work we give account of the identification of one of these components (x_4) on the basis of GC-MS studies and repeated gas chromatographic analyses carried out after isolation by paper chromatography. Component x_4 was identified by these analyses as 3α,20α-dihydroxy-5β-pregnan-11-one. The excretion values of the identified component in infants and children of different ages with C_{21}-hydroxylase deficiency are also reported.

Reference steroids: These were kindly supplied by Prof. D. N. Kirk (Medical Research Council, Steroid Reference Collection, London).

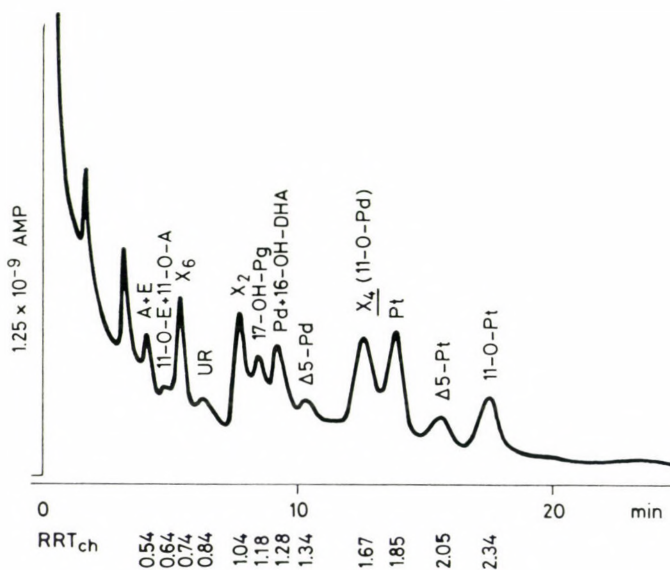

Fig. 1. Gas chromatogram of urinary steroids as acetyl derivatives obtained from a 12 years old girl with C_{21}-hydroxylase deficiency. Conditions: enzyme hydrolysis; 3% Sp 2100 column; column temp.: 250°C; detection: FID x_2, x_6 are unidentified peaks

Material studied: In order to identify component x_4, a steroid extract was prepared from the 24-hour urine of T. K., a 12 years old girl with C_{21}-hydroxylase deficiency by the method described in our publication cited in the Introduction:

a) three-tenth of the extract was hydrolyzed by acid and extracted by toluene,

b) two-tenth was submitted to β-glucuronidase-arylsulphatase hydrolysis and ether extraction,

c) one-tenth was hydrolyzed with acid and extracted by toluene, processing in two separate steps the two extracts marked "pH 1 fraction" and "glucuronid fraction".

In addition to the material above, the amount of component x_4 was quantitatively determined in the case of 7 salt-loosing infants and children with C_{21}-hy-

droxylase deficiency, whose clinical data and steroid diagnosis are given in detail in our previous publication (Kecskés et al., 1981).

Analytical methods: An aliquot of the steroid extract from the urine of T. K., a 12 years old patient, hydrolyzed enzymatically or by acid, was fractionated on Schleicher-Schüll 2043) a paper in Bush B_3 system. On the reference strip androsterone, 11-keto-androsterone and 11-hydroxy-etiocholanolone were run. The reference strip was detected by Zimmermann's reagent and the strip containing the steroid extract was divided into 5 zones without detection, according to the following principles:

fraction I: steroids more mobile than androsterone
fraction II: steroids more mobile than 11-keto-etiocholanolone
fraction III: steroids with mobilities higher than and equal to that of 11--hydroxy-etiocholanolone
fraction IV: steroids more polar than 11-hydroxy-etiocholanolone
fraction V: steroids remaining at the start.

The zones were eluted by a 1 : 1 mixture of ethanol and benzene. The dried eluates were analyzed by gas chromatography on an Sp 2100 column. In the analysis of Fraction IV containing x_4, the following additional chemical reactions were carried out:

a. gas chromatography or GC-MS analysis after acetylation;
b. gas chromatography, without forming a derivative
c. gas chromatography, or GC-MS analysis after oxidation by chromium trioxide;
d. gas chromatography, after reduction of the acetylated component x_4 by sodium borohydride and repeated acetylation.

Oxidation by chromium trioxide was carried out by 1% chromium trioxide dissolved in 96% acetic acid. An aliquot of Fraction IV was dissolved in 0.2 ml of glacial acetic acid, and after the addition of 0.2 ml of the reagent the mixture was kept in an ice-bath for 1 hour. 2 ml of 20% ethanol was then added, followed by extraction by 2 ml benzene. The benzene extract was washed with 8% sodium hydrocarbonate and with distilled water, dried with sodium sulphate; the solvent was then evaporated and the material analyzed by gas chromatography.

Reduction by sodium borohydride was done by 1.5% sodium borohydride in 87% ethanol. An aliquot of Fraction IV was dissolved in 0.2 ml of ethanol and to it 0.2 ml of reagent was added. The mixture was left at room temperature for 1.5 hours. Following the addition of 10 μl of glacial acetic acid it was placed in a 50 °C water bath for 15 minutes, then made up to 2 ml with distilled water and extracted by dichloromethane. After washing with water and evaporation, the material was acetylated and analyzed by gas chromatography.

GC-MS analyses were carried out on Fraction IV of urine extracts hydrolyzed enzymatically or by acid and separated by paper chromatography, on a Micromass MM-12-F1A magnetic mass spectrometer linked to a Pye-104 gas chromatograph, under conditions described earlier (Kecskés et al., 1980).

Results

On the chromatogram of the enzimatically hydrolyzed urine from T. K., a 12 years old girl with C_{21}-hydroxylase deficiency, the disease is indicated by the presence of 17-hydroxy-pregnanolone (RRT_{ch} = 1.18), pregnanetriol (RRT_{ch} = = 1.85) and 11-keto-pregnanetriol (RRT_{ch} = – 2.34). In addition to these well-known components, the nonidentified components x_2 (RRT_{vh} = 1.04) and x_4 (RRT_{ch} = 1.67) were also observed; the latter of these was eluted immediately

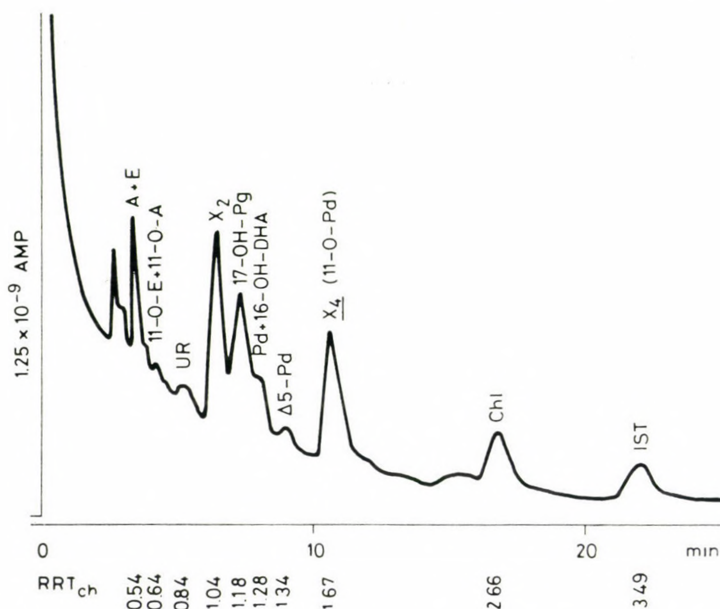

Fig. 2. Gas chromatogram of unirary steroids as acetyl derivatives obtained from a 12 years old girl with C_{21}-hydroxylase deficiency. Conditions: acid hydrolysis; 3% Sp 2100 column, column temp.: 250°; detection: FID · x_2 is an unidentified peak

before pregnanetriol. Both nonidentified components exhibited heat and acid stability (Fig. 2).

Fraction of the paper chromatogram contained component x_4 and also a small amount of component x_2 (Fig. 3).

Fraction IV, isolated by paper chromatography, was subjected to GC-MS analysis. Component x_4 obtained, from urine hydrolyzed either enzymatically or by acid gave identical mass spectra (Fig. 4a). The molecular weight of the compound is M = 418, and its mass spectrum demonstrates the presence of two acetyl groups (m/e 358 and 298). As a reference, 3α,20β-dihydroxy-5β-pregnan-11--one-diacetate, eluted near component x_4 (RRT_{ch} = 1.54), $I^{250°}_{Sp\,2100}$: 2980) was

available to us, and the mass spectrum of this steroid recorded under identical conditions proved to be identical with that of component x_4 (Fig. 4b).

The discernibility of x_4 from the reference steroid by mass spectrometry and the slight difference between the retention times of the two compounds indicated that there is only a small, presumably stereochemical difference between them. In order to elucidate this difference, the functional groups of the two compounds were subjected to various chemical reactions: acetylation, reduction of the

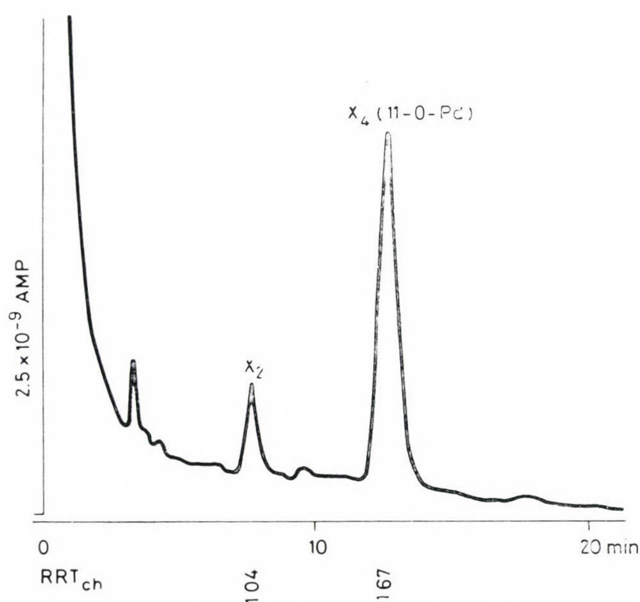

Fig. 3. Gas chromatogram of Fraction IV as acetyl derivatives obtained by paper chromatography from the acid hydrolyzed urine of a 12 years old girl with C_{21}-hydroxylase deficiency. Conditions: 3% Sp 2100 column; column temp.: 250°; detection: FID · x_2 is an unidentified peak

acetylated derivative by sodium borohydride and repeated acetylation, furthermore oxidation of the free steroids by chromium trioxide. The gas chromatographic data obtained after modification are listed in Table 1. The reference 3α,20β-dihydroxy-5β-pregnan-11-one was compared with the reference 5β-pregnan-3α,11β,20β-triol and with the structurally similar reference 5β-pregnan-3α,20β-diol which is not oxygenated on C_{11}. It was established that the 11-keto substituent increases the retention index by 110 units and the 11β-hydroxyl substituent by 155 index units.

The non-derivatized form of component x_4 has a retention increment over the reference 5β-pregnan-3α,20α-diol. After the acetylation of component x_4, the

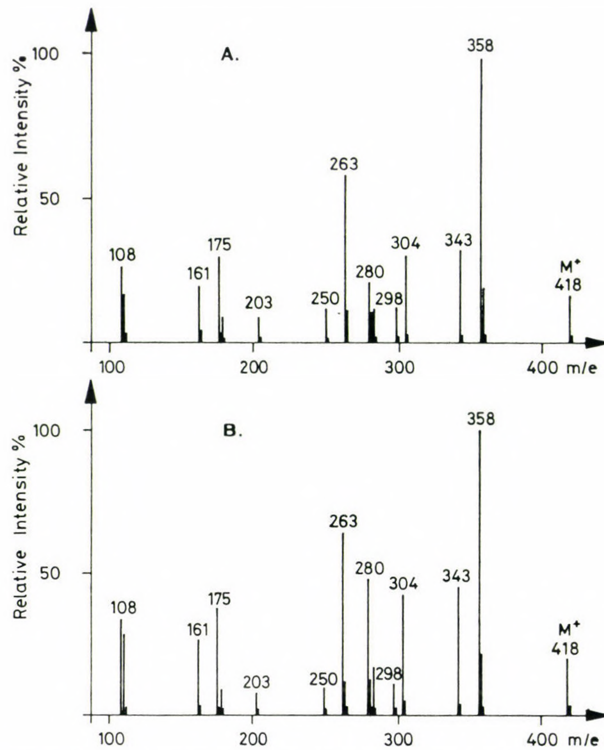

Fig. 4. Mass spectrum of the acetyl derivative of component "x_4" (A) and 3α, 20β-dihydroxy-5β-pregnan-11-one (B)

increment is 100 index units as compared to the reference 5β-pregnan-3α,20α-diol--diacetate, i.e. it is essentially unaltered. The increment by 100–120 index units of component x_4, both in its free form and as an acetylated derivative, over the reference 5β-pregnan-3α,20α-diol corresponds to the increment of the 11-keto group. When component x_4 was reduced and repeatedly acetylated, the increment was increased only by 45 units, which means that reduction produced a blocked hydroxyl group that could not be acetylated. These data suggest that the third substituent of component x_4 is a keto group situated on carbon atom[11]. Owing to the 20-hydroxyl group the retention index of the reference 5β-pregnan-3α,20α-diol and of its acetylated derivative is 25–30 units higher than that of the 20β--hydroxyl isomer. The retention value of both free and acetylated component x_4 is higher by the same value as that of the reference 3α,20β-dihydroxy-5β-pregnan--11-one. In order to confirm the 5 "β"-pregnane structure, an aliquot of non-derivatized Fraction IV was oxidized by chromium trioxide and compared to the reference 3α,20β-dihydroxy-5β-pregnan-11-one treated in the same way. The gas chromatographic retention data were identical, just as the mass spectra obtained by GC-MS analysis (cf. Figs 5a and 5b).

Table 1

Changes in the retention index of component x_4 and of the reference steroids after chemical reactions (derivatization, reduction, oxidation) and the calculated retention increments of the 11-oxygenated functional group

No.	Steroid (or *functional group*) used for the calculation of retention imcrement	Free		Acetylated		Reduced + + acetylated		Oxidized	
		RRT	$I^{250°}_{Sp\ 2100}$	RRT	$I^{250°}_{Sp\ 2100}$	RRT	$I^{250°}_{Sp\ 2100}$	RRT	$I^{250°}_{Sp\ 2100}$
1	5 β-pregnane-3 α,20 β-diol	0.62	2645	1.18	2870				
2	3 α,20 β-dihydroxy-5 β--pregnane-11-one	0.84	2755	1.54	2980	1.82	3025	0.84	2755
	(retention increment of 11-keto group)		110		110				
3	5 β-pregnane-3 α,11 β,20 β-triol	0.96	2800	1.82	3025			0.84	2755
	(retention increment of 11 β-hydroxy-group)		155		155		155		
4	5 β-pregnane-3 α,20 α-diol	0.64	2660	1.28	2900				
5	"x_4"	0.92	2780	1.67	3000	1.94	3045	0.84	2755
	(retention increment of functional group of "x_4" at C_{11})		120		100		145		

The molecular weight and fragmentation of the components correspond to the triketo-pregnan structure expected after chemical transformation.

All these data prove that component x_4 possesses a 5 "β"-pregnan skeleton and that the three functional groups are situated on the same carbon atoms as in the reference steroid.

Quantitative determination

11-Keto-pregnanediol excretion was quantitated in 12 determinations on 7 infants and children with C_{21}-hydroxylase deficiency; the data are shown in Table 2. At the time of the investigation the patients were aged from 4 months to 13 years and, with the exception of 3, they all received substitution corticoid treatment. In the case of infants the excretion of 11-keto-pregnanediol was low, between the ages of 1 and 13 years its absolute value was manifold increased as compared to infants; however, it proved to be highly variable. The extent of steroid excretion in C_{21}-hydroxylase deficiency depends on the effectiveness of substitution corticoid therapy, therefore a clearer view is obtained of the excretion of 11-keto-pregnane-diol if its amount is compared to that of total steroids or to the other 11-hydroxyl-ated steroid, 11-keto-pregnanetriol and is given as the percentage of these. In calculating these two percentage values, we used the corresponding data from our previous publication (Kecskés et al., 1981).

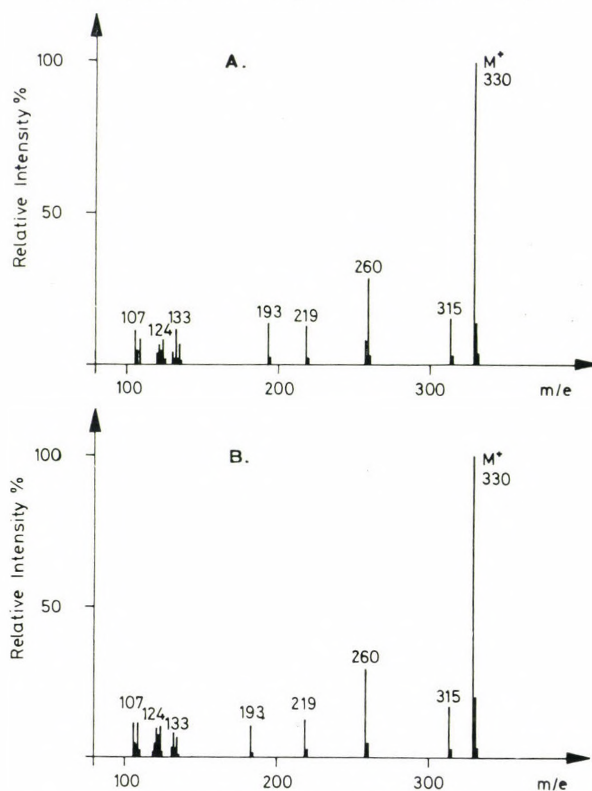

Fig. 5. Mass spectrum of the underivatized component "x_4" after oxidation by chromium trioxide (A) and 3α, 20β-dihydroxy-5β-pregnan-11-one after oxidation by chromium trioxide (B)

Table 2

Absolute and relative values of 11-keto-pregnanediol excretion for 7 infants and children with C_{21}-hydroxylase deficiency

Case	Age year (week)	Sex	Corticoid treatment mg/day	11-O-Pd μg/day	Percentage of 11-O-Pd excreted with reference	
					to total steroids	to 11-O-Pt
O. I.	(4)	M	none	trace	not calculated	not calculated
O. I.	(6)	M	12.5	trace	not calculated	—
M. V.	(8)	F	none	54	0.8	—
K. Z.	(10)	M	none	50	1.0	5.3
K. T.	1 1/2	M	5	820	2.3	38.0
K. T.	4	M	5	294	2.4	18.2
I. T.	2 1/2	M	5	280	1.0	10.2
I. T.	3 1/2	M	2.5	828	4.6	—
V. Z.	6	M	5	960	5.3	60.0
T. K.	11	F	20	880	5.8	27.0
T. K.	12	F	20	1080	7.1	77.0
T. K.	13	F	10	420	17.0	—

The amount of 11-keto-pregnanediol relative to total steroids is insignificant in infants (1% or less), between the ages of 3 and 6 years it shows an increasing tendency (2.3–5.3%), while the highest value found in puberty is 17%. 11-Keto--pregnanetriol was determined in 8 cases. The excretion of 11-keto-pregnanediol relative to 11-keto-pregnanetriol during infancy is 5.3% or lower, in children before puberty it is 10.2–60% while in puberty in one case it amounted to 77%.

In the urine processed by two-step hydrolysis of T. K., a 12 year-old girl with C_{21}-hydroxylase deficiency, 38% of the excreted 11-keto-pregnanediol was found in the "pH 1 fraction" and 62% in the "glucuronid fraction".

Discussion

In C_{21}-hydroxylase deficiency the 11β-hydroxylation of 17-hydroxy-progesterone is intensive in the adrenals, which is indicated by the high amount of 11-keto-pregnanetriol excreted in the urine. This "pathological" steroid is not detectable in healthy individuals, and therefore its presence is one of the most reliable diagnostic signs of C_{21}-hydroxylase deficiency (Finkelstein et al., 1953). Under physiological conditions, in the human adrenal gland the hydroxylation steps from pregnenolone to cortisol follow each other in the order of $C_{17}-C_{21}-C_{11}$ (Levy et al., 1954; Pasqualini et al., 1964; Maschler et al., 1977); in other species, however, C_{21} deoxysteroids may also be hydroxylated on C_{11} and may be the substrates of C_{21}-hydroxylation. 11-Hydroxy-progesterone secretion has been shown in the venous blood in canine (Oertel, Eik-Nes, 1962), and porcine (Heap et al., 1966) adrenals. Its amount as compared to other steroids was about 1/15th of glucocorticoid secretion and equalled 1β-hydroxy-androstendione secretion.

One of the metabolites of 11-hydroxy-progesterone is 11-keto-pregnanediol, identified by Mason (1948) in the urine of an Addison patient treated with 11--dehydrocorticosterone, and by Lieberman et al. (1950) in the urine of a patient with congenital adrenal hyperplasia. The latter authors mention without numerical data that 11-keto-pregnanolone is detectable in the urine collected for several days, of healthy individuals. Fleetwood et al. (1978) studied the excretion of 11-keto-pregnanolone, another metabolite of 11-hydroxyprogesterone; they could not detect this compound in healthy persons. They could, however, trace it in small amounts in hirsutism and correlated its occurrence in these patients with partial 21-hydroxylase deficiency.

In the course of the study of the steroid excretion of a child with C_{21}-hydroxylase deficiency, Fukushima et al. (1957) found 1 mg of 11-keto-pregnanediol in the urine after combined enzymatic and pH 1 hydrolysis. The relative amount of 11-keto-pregnanediol was 0.7% as compared to the amount of total steroids and 8.3% relative to 11-keto-pregnanetriol.

In our material, the relative value of 11β-hydroxylation of $C_{21}O_2$ steroids was similar to the above-mentioned single observation in the case of infants; in children we could detect multiple amounts. Our data indicate that with the

advancement of age, the 11β-hydroxylation of progesterone is increased. It must be borne in mind, however, that all children studied received substitution corticoid treatment. If cortisol possesses an intraglandular regulatory role, exogeneous corticoids may have affected the excretion relative to pregnanetriol by way of decreasing 17α-hydroxylase activity. According to our investigations, in children with 21-hydroxlase deficiency the sequence of hydroxylation may differ from normal and 11β-hydroxylation of progesterone may be significant.

The authors are indebted to Prof. D. N. Kirk (Medical Research Council, Steroid Reference Collection, London) for making available for us the reference steroids, to Dr. József Tamás (Central Research Institute for Chemistry of the Hungarian Academy of Sciences, Budapest) for his help in the evaluation of the GC-mass spectrometric measurements, and to Mrs. Márta Szécsényi for her enthusiastic technical assistance.

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Sliding Theory: Facts and Texts

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In his book "Reflexions on Muscle" A. Huxley (1978), defending the sliding theory and referring to my paper "Sliding Friction contra Sliding Hypothesis" (Ernst, 1977)* declares (p. 66): "(the latter) contains an error; if correct it would create severe difficulties for sliding filament theories of all kinds." A survey of the pertinent literature may help to clarify which of the two publications contains more errors or statements not sufficiently based on facts.

Introduction

My short paper emphasized three facts:

A. The sliding theory postpones most of the *activity processes* to the data of the microstructure of the muscle and describes also even that very one-sidedly.

B. The force of the viscous resistance is given in my paper as

$$F = \eta q \frac{\Delta v}{\Delta r},$$

where η = viscosity of the interfilamentous medium, q = *the total sliding surface*, and v = the velocity gradient, v being the velocity of the sliding motion, r = the distance between the sliding surfaces (here q is very large, r is very small). Computing the values of these factors for an (imaginary) frog muscle 3 cm in length and 1 cm^2 in cross section will reveal the sliding resistance to be greater than even the maximal force attainable by this muscle in a tetanus.

C. In the case of spontaneous relaxation the sliding resistance is doubtless the same as at shortening; therefore, it arises the question where the force is taken from to overcome this viscous resistance opposing the reversal of the sliding motion backward.

* Comment to J. Tigyi's lecture entitled "Biophysics of Muscle Contraction" delivered at the 1st European Expert Committee Meeting on "Perspectives on Biophysics", Budapest, June 2—4, 1976.

Statements in A. Huxley's book concerning points A, B, C

A. 1. As to the microstructure of the striated muscle in A. Huxley's book, E. Huxley's figure (p. 37) is accepted, according to which the thick filaments are floating inside the sarcomere in contrast to the thin filaments bound to the Z-discs.

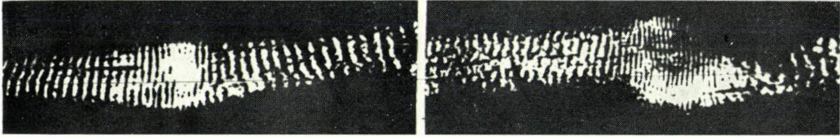

Fig. 1. Contractions of a cross-striated muscle fibre in polarized light (Hydrophilus). ×300

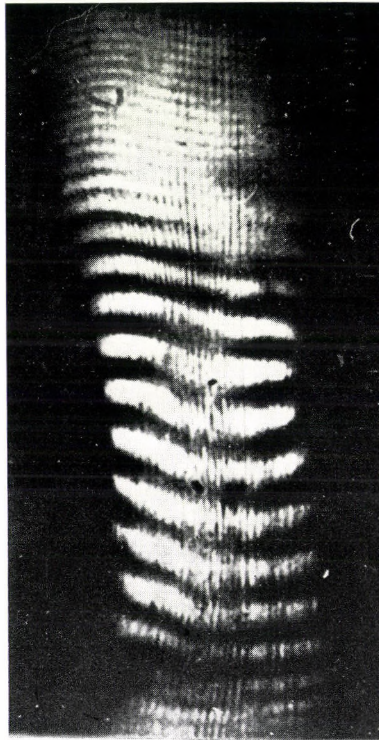

Fig. 2. Contraction of a fibre (muscle from the head of Vespa, above) in polarized light. ×650

In this book (p. 65): "... each thick filament is thought ... sliding within a cylinder ... formed by the thin filaments ..." In this sense: a) Either the whole thick filament should move towards one of both Z-discs. Therefore the question: what is the factor deciding the *direction* of the movement of the thick filament,

and how are all thick filaments in a sarcomere coordinated to move towards the same Z-disc. b) Or the thick filaments should divide into two parts, each half migrating toward the opposite Z-disc of a sarcomere, and later the two halves should unite again.*

2. The book "Reflexions on Muscle" is engaged in describing the microstructure of muscle in the size of about one third of the whole book and contains many microphotographs, but not a single one which would serve as a basis of his sliding theory.** All the more becomes conspicuous the endeavour to neglect,

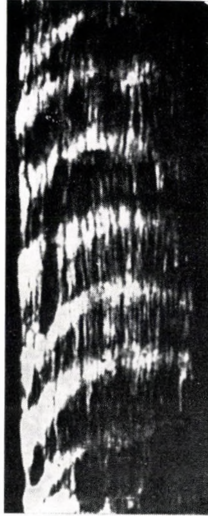

Fig. 3. Fibre (frog gastrocnemius); after rigor (a. c.) stretched, in polarized light. × 370

or conceal or refute data opposing the sliding theory.

Especially, the decrease in the width of the A-bands in contracting sarcomeres is repeatedly strongly denied; therefore, Fig. 1 should stand here showing a muscle fibre contracted spontaneously; the A-bands are bright in polarized light. The width of the sarcomeres and that of the A- and I-bands are smaller in the shortened than in the resting parts.

Furthermore, W. J. Schmidt, one of the best known experts in birefringence of biological tissues, is not even mentioned: Figure 62 in his book (1937, p. 201)

* Another assumption according to which the thin filaments are moving from both Z-discs of a sarcomere towards the thick filaments would mean that thin filaments continuously infiltrating along the length of a fibril would be directed in their movement by the thick filaments lacking of any structural connection with the body of the fibril and floating in the interfilamentous fluid of the sarcomeres. The plausibility of such a process is questionable.

** At any rate, I myself also could hardly find two equal pictures published by followers of the sliding theory, not to mention many other research workers.

is reproduced here (Fig. 2). This shows without doubt that the width of the sarcomeres and the A-bands inside them are very much decreased in a contracted part of a fibre. That decidedly contrasts to the sliding theory.

Likewise, A. Huxley's book fails to mention W. J. Schmidt's paper (1937)* from which Fig. 3 (p. 736) is reproduced here (Fig. 3); it shows a contracted and afterward stretched fibre in polarized light: the contraction bands and long parts of fibrils are birefringent.

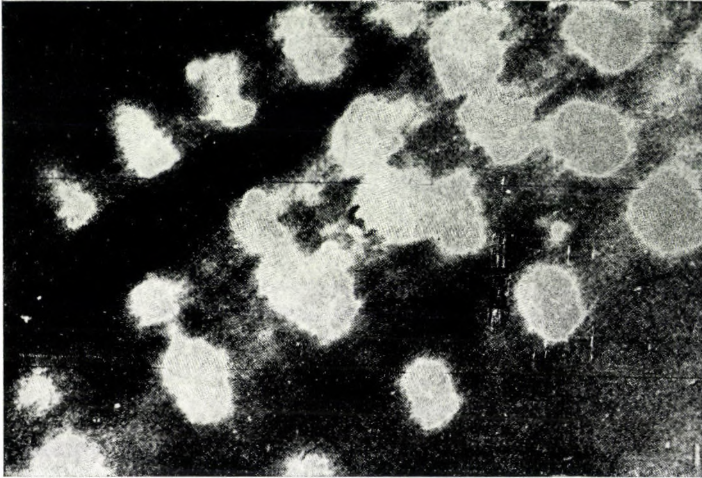

Fig. 4. Single fibrils (flight muscle, Hydrophilus) strongly stretched, in polarized light. ~ ×3000

This does not fit at all into the sliding theory, similarly to the fact (Ernst et al., 1956) that single fibrils (wing muscles of Hydrophilus) stretched very much contain (Fig. 4) a) A-bands of greatly *increased birefringence* and b) *birefringent filaments* extending without interruption *through many sarcomeres.* That is: Positively birefringent filaments are shown between neighbouring A-bands in a stretched sarcomere. These experimental facts are in contrast with the microstructure and the processes in it as described by A. Huxley's sliding theory.

3. The epilogue of A. Huxley's book emphasizes doubt about the reality value of the electron microscopic data of today; with that I agreed in the past (see e.g. Ernst, 1963; Achátz, 1968) and agree also today (see chapter B, p 46), in contrast to the one-sidedness with which the literature of the microstructure is dealt with in this book. Furthermore, this work seems to believe itself to be justified in changing the historical truth when it states (p. 30): "Lundsgaard's

* Immediately after our paper (Ernst, Kellner, 1936) had been published, prof. Schmidt asked for some muscle preparations described by us, (Fig. 3 shows one of our preparations in polarized light).

famous experiment, published in 1930 . . . disproved" . . . the lactic acid theory of muscle contraction. The truth sounds (e.g. Ernst, 1963, p. 17): "The data of Schwartz and Oschmann(1925) on lactic-acid-free contraction of muscle treated with *bromacetic acid* . . . five years later the lactic-acid-free contraction of muscle treated with *iodoacetic-acid* found by Lundsgaard (1930) . . . (see Verzár, 1943; Bethe, 1952)."

A question as epilogue to A: In which statements by both of us does the reader find more errors?

B. 1. The formula for the viscous resistance to the sliding of each thick filament is (in A. Huxley's book, p. 66):

$$"2\pi\eta(v)ln(a_2/a_1)\ ,$$

where η = the viscosity of the fluid, l = the length of the overlap zone, and v = the relative speed of sliding (a_1 = radius of the thick filament, a_2 = radius of a cylinder formed by the thin filaments which surround it)."

As to this statement I cannot help asking who is supposed to accept that

a) the thick filament is sliding within the cylinder of "*the*" thin filaments (of how many filaments), and not the sliding of many more thin filaments?

b) v = the "relative" speed; speed = velocity = celerity is in physical meaning always relative. In the sliding resistance, however, not the "relative speed" is decisive but the *velocity gradient* in which also the *width of the fluid migrating together with the sliding body* is determinative.

c) the formula of the force of the viscous resistance begins (in A. Huxley's book) with 2π; it is not clear to which magnitude it belongs: surely not to η, not to l, not to v, and also not to a_2/a_1 because this latter does not mean a real value with dimension, but a simple ratio.

2. According to A. Huxley's book (p. 67): "Frog muscle at room temperature, η is probably about 2×10^{-3} Pa.s" (=2 centipoise = 0.02 $gcm^{-1}s^{-1}$); the interfilamentous fluid being considered a solution of globular proteins at a concentration of about 0.2 g/ml, and the viscosity of water as half of this value.

a) The viscosity of a fluid in bulk must not be considered to be equal to that of the same fluid in a very thin film; thus (Derjagin, Samigin, 1941, p. 62) e.g. the viscosity (η) of water (16 – 18 °C) if it is a film of the width (w)

w (nm)	η (Poise unit)	= 0.1 Pa · s
180	0.0098	10^{-3} ,,
20	0.0450	4.5×10^{-3} ,,

In the case of the sliding filament the width of the fluid film moving with the filaments is much smaller than 20 nm; at this distance there is the opposite filament also binding a fluid layer similar to that of the sliding filament and therefore also the viscosity should increase. Furthermore, the interfilamentous fluid is certainly more viscous than a clear solution.

b) In A. Huxley's book (p. 67): "The maximum shortening speed . . . v – the shortening speed per half sarcomere – is about 10 μm/s." *In reality*, the shortening velocity of a frog muscle (v_m) is *measured* to be approximately 1/3–1/2 of its resting length in the time of about 50 ms; e.g. for a gastrocnemius of 3 cm resting length $v_m = 1$ cm/50 ms or $v_m = 10^4$ μm/50 ms. The afore-mentioned value for a whole sarcomere $v_s = 20$ μm/s $= 20$ μm/1000 ms, $v_s = 1$ μm/50 ms. Consequently, the real distance made by the shortening muscle, being 10^4 times larger than that of a sarcomere, can result only if the *shortening of all sarcomeres* in the whole length of the muscle *becomes summed up* (they shorten of course simultaneously). Thus, since the sliding process takes place simultaneously in all sarcomeres in the whole length of the muscle, the viscous resistance will be effective in all of them, i.e. it should become summed up in a real shortening of a real muscle – if the sliding theory itself agreed with reality.

Result of point B

Not my paper contains the error ascribed to it by A. Huxley's book, but the latter one, taking into account the viscous resistance in the shortening sarcomeres lying but *in a single cross section* of the muscle and disregarding those the shortening of which, summing up, brings about the real shortening of the whole muscle.

C. Quotation of the last paragraph of my paper (p. 85) containing the "error" ascribed to it by A. Huxley's book: "As to the *relaxation* of the shortened muscle there is no doubt that the sliding friction is effective in the same manner as during shortening. Therefore the question arises where the force is taken from to *overcome the viscous resistance opposing the reversal of interdigitation.*"

In agreement with the well balanced and wide-ranging expoundings in the Epilogue (being in contrast with the apodictic statements in the whole 96 pages of A. Huxley's book) I wish to propose some self-restraint in producing muscle-theories or even hypotheses concerning the mechanism of bioprocesses – at least today. Namely, according to the physics of the 19th century, the material world was considered to produce processes by different linkages of atoms regarded as given unchanged for ever. On this basis chemistry and biochemistry produced very valuable results, (and biophysics was lagging behind them). The 20th century, however, beginning with Bequerel's discovery, is on the way to get an insight into the processes of the material world on a subatomic level, i.e. processes between the elementary particles and in radiations accompanied by them. On this basis the following generation will probably be able to found biology on an exact scientific level by research teams consisting of experts of exact sciences and led by biophysicists.

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Bound Potassium in Muscle III

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In contrast with resting muscles which lost 11 per cent of their original K content and 43 per cent of their original Na content in glycerol (Hummel, 1980), stimulated muscles behaved quite differently: they lost more potassium and less sodium. The results suggest that, during excitation, bound potassium can be exchanged for sodium originated from the extracellular space. On the basis of the entropy change of bound water and bound alkalis a new hypothesis of biological excitation is put forward. According to it biological excitation can travel by phonon transport *via* K biocomplexes.

Introduction

What is the state of potassium in muscle like: is the ion bound or freely dissolved? The question has not yet been settled unequivocally. If potassium in muscle is in a bound state, and if it is released in the course of excitation, more free K^+ should be found in the stimulated muscle than in its resting pair.

Higher potassium mobility was found in overstimulated muscles than in normal ones (Hummel, 1978).

Ernst and Scheffer (1928) determined the normal potassium content of frog muscles in each of the four seasons over several years and found it to be 3.0 ± 0.3 mg/g of fresh frog muscle. This value remained practically unchanged when the muscle was perfused with normal Ringer solution for several hours. However, when the perfused muscle was *directly* stimulated by a.c. several times (for 0.3 to 0.4 sec in every 2 sec) until fatigue, the potassium content decreased by about 30 to 50 per cent, and a significant Na gain was found in the stimulated muscles. In contrast, these authors failed to see either K loss or Na gain in muscles stimulated *indirectly* [just as did later also Ernst and Csúcs (1929)]. Thus, there is a K – Na interrelation in muscle.

The resting muscles were investigated by means of glycerol treatment; and the glycerol treatment proved to be a useful method for determining the freely dissolved ion content of muscle (Hummel, 1980). Thus, by determining the K content of the resting and of the stimulated muscle treated with glycerol one can answer the question of how much potassium is released during excitation under the given experimental conditions.

Materials and methods

M. sartorius and m. semimembranosus of *Rana esculenta* were used. The sartorius muscles of a frog were dissected and weighed. One of them was put into a plexi box, and fixed by two thread loops fastened to its ends. The other one was put into a glass tube, and similarly fixed at its resting length. Sartorii of other three frogs were prepared in the same way. Four sartorius muscles were kept in the closed box and the other four ones in four closed glass tubes. To prevent drying of muscles wet wadding paper was put on the bottom of the box; also the glass tubes had humid air. Electrodes were fixed to the ends of the muscles in the box. The connections of muscles were parallel; the same voltage was applied to each sartorius for 0.1 second. Stimulation was started with 2V and lasted until the muscles exhausted. Thereafter, the voltage was increased. The following voltages were used for direct stimulation until fatigue: a.c. of 2, 4, 6, 8, 10, 12, 14, 16, 20 and 110 V. The whole stimulation procedure took about half an hour.

In the experiments performed with semimembranosus muscles only one frog was prepared, so one semimembranosus muscle was stimulated in the plexi box and its pair served as resting control. The stimulation procedure lasted about the same time as in the case of sartorius muscles.

The stimulated muscles and their control pairs were put into cooled (+2 °C) glycerol of 87 weight per cent. All sartorius muscles were kept in glycerol (+2 °C) for one hour, and the semimembranosus muscles for one and a half hour. After glycerol treatment the muscles were blotted by absorbent paper and weighed.

Both the stimulated and the control muscles were dried at 120 °C for 12 hours and ashed at 450 °C overnight. The dried muscles and the ashes were weighed. The potassium and sodium content of the ashes were determined by flame photometer.

Results

Less potassium and more sodium was found in the stimulated than in the unstimulated muscles after the glycerol treatment. The results obtained on sartorius muscles are shown in Table 1 .The difference between K content of stimulated and unstimulated muscles is significant at a level of $P = 0.05$. There is also a significant difference between their Na content at a level of $P = 0.01$.

The data of glycerol-treated semimembranosus muscles are presented in Table 2. It also shows a significant K loss in stimulated muscles when compared with their resting pairs. Summarizing the data (Table 3) it can be seen that, beside the same water loss, the stimulated sartorius muscles lost more potassium; similar results were obtained in semimembranosus muscles. It is also unambiguous that more residual sodium is present in the stimulated muscles than in their resting pairs.

Table 1

Stimulated (s) and unstimulated (u) sartorius muscles of frog treated with glycerol. Fresh weight of four sartorii (G_f); weight of the same four muscles after glycerol treatment (G_g); dry weight (G_d); ash weight (G_a); potassium content (K); sodium content (Na)

No.		g			mg		
		G_f	G_g	G_d	G_a	K	Na
1	s	0.67	0.23	0.14	3.7	1.86	0.29
	u	0.65	0.21	0.13	3.6	2.43	0.21
2	s	1.00	0.35	0.27	4.5	1.68	0.42
	u	1.00	0.33	0.25	6.0	2.13	0.27
3	s	0.74	0.27	0.17	4.7	1.59	0.35
	u	0.72	0.26	0.16	4.5	1.95	0.26
4	s	0.90	0.34	0.22	6.5	2.16	0.37
	u	0.90	0.33	0.22	6.2	2.43	0.28
5	s	0.82	0.29	0.18	5.0	1.83	0.28
	u	0.84	0.29	0.19	5.3	2.13	0.19
6	s	0.67	0.25	0.16	6.9	1.50	0.28
	u	0.67	0.23	0.15	5.0	1.80	0.20
7	s	0.60	0.22	0.14	6.6	1.29	0.23
	u	0.61	0.22	0.14	7.1	1.47	0.18
8	s	0.76	0.28	0.18	7.4	1.92	0.33
	u	0.74	0.27	0.17	8.0	2.10	0.24
9	s	0.57	0.20	0.11	6.4	1.08	0.21
	u	0.54	0.18	0.11	6.8	1.23	0.13
10	s	0.71	0.27	0.17	4.3	1.68	0.28
	u	0.73	0.28	0.18	4.7	1.80	0.26
11	s	0.78	0.27	0.17	8.4	1.98	0.33
	u	0.78	0.26	0.17	8.8	2.34	0.23
12	s	0.59	0.19	0.13	5.8	1.56	0.23
	u	0.59	0.18	0.13	5.3	1.77	0.17
13	s	0.79	0.28	0.18	9.2	2.07	0.35
	u	0.77	0.26	0.18	9.5	2.34	0.22
14	s	0.70	0.25	0.16	2.9	1.83	0.24
	u	0.70	0.25	0.16	4.0	1.98	0.18
15	s	0.51	0.19	0.12	2.0	1.20	0.22
	u	0.51	0.18	0.12	2.4	1.41	0.14
16	s	0.75	0.24	0.14	7.6	1.77	0.30
	u	0.75	0.24	0.15	7.7	2.19	0.20
17	s	1.06	0.37	0.22	8.2	2.82	0.35
	u	1.04	0.37	0.22	10.0	3.09	0.23
18	s	0.98	0.30	0.20	7.6	2.40	0.26
	u	0.98	0.32	0.21	9.5	2.76	0.19
19	s	1.00	0.34	0.21	8.0	2.49	0.30
	u	1.03	0.34	0.22	8.9	2.85	0.24
20	s	0.95	0.33	0.20	7.0	2.25	0.27
	u	0.95	0.32	0.20	7.8	2.76	0.19
21	s	0.87	0.32	0.21	7.5	2.40	0.36
	u	0.88	0.31	0.21	7.8	2.64	0.27
22	s	0.89	0.30	0.19	6.3	2.22	0.37
	u	0.90	0.31	0.19	7.7	2.82	0.23
23	s	0.65	0.21	0.14	2.9	1.78	0.29
	u	0.65	0.21	0.14	3.9	2.03	0.16
$\bar{x} \pm \sigma_s$		0.78 ± 0.03	0.27 ± 0.01	0.17 ± 0.01	6.1 ± 0.4	1.89 ± 0.09	0.30 ± 0.01
$\bar{x} \pm \sigma_u$		0.78 ± 0.03	0.27 ± 0.01	0.17 ± 0.01	6.5 ± 0.4	2.19 ± 0.10	0.21 ± 0.01

Table 2

Stimulated (s) and unstimulated (u) semimembranosus muscles of frog treated with glycerol. Fresh weight of a semimembranosus (G_f); weight of the same muscle after glycerol treatment (G_g); dry weight (G_d); ash weight (G_a); potassium content (K); sodium content (Na)

No.		g			mg		
		G_f	G_g	G_d	G_a	K	Na
1	s	1.05	0.47	0.23	9.1	3.06	0.32
	u	1.04	0.50	0.24	10.3	3.45	0.21
2	s	0.97	0.49	0.23	7.7	3.12	0.35
	u	0.99	0.53	0.23	9.0	3.45	0.29
3	s	0.74	0.32	0.17	7.2	2.28	0.31
	u	0.75	0.32	0.17	7.4	2.50	0.25
4	s	0.91	0.47	0.23	6.3	2.82	0.34
	u	0.91	0.46	0.23	7.6	3.03	0.24
5	s	0.79	0.41	0.19	5.7	2.31	0.25
	u	0.79	0.42	0.19	5.8	2.73	0.19
6	s	0.97	0.49	0.23	8.5	2.13	0.37
	u	0.96	0.51	0.23	9.9	2.70	0.30
7	s	1.22	0.57	0.29	10.4	3.29	0.21
	u	1.23	0.58	0.27	11.3	3.85	0.14
8	s	0.75	0.36	0.19	8.1	2.10	0.22
	u	0.73	0.33	0.19	8.6	2.33	0.12
9	s	1.16	0.52	0.25	9.2	2.70	0.14
	u	1.15	0.50	0.26	10.1	3.03	0.10
10	s	0.87	0.33	0.23	5.7	2.00	0.14
	u	0.88	0.34	0.23	6.4	2.20	0.10
11	s	0.72	0.27	0.15	3.3	1.65	0.30
	u	0.74	0.29	0.15	4.3	2.13	0.25
12	s	1.00	0.41	0.21	8.1	2.76	0.37
	u	1.05	0.46	0.23	9.2	3.06	0.31
13	s	0.68	0.33	0.16	6.2	1.53	0.12
	u	0.72	0.31	0.15	6.1	1.85	0.09
14	s	1.15	0.54	0.25	8.0	2.94	0.28
	u	1.12	0.53	0.25	9.8	3.36	0.17
15	s	0.67	0.30	0.13	4.3	1.48	0.13
	u	0.68	0.27	0.13	4.4	1.73	0.08
16	s	0.82	0.36	0.18	9.3	2.38	0.20
	u	0.82	0.39	0.18	9.0	2.73	0.15
17	s	0.72	0.36	0.17	2.8	1.75	0.16
	u	0.72	0.34	0.16	3.0	1.98	0.10
18	s	0.67	0.32	0.16	5.2	1.63	0.22
	u	0.66	0.31	0.15	5.8	1.78	0.16
19	s	0.71	0.29	0.13	4.4	1.30	0.12
	u	0.73	0.29	0.13	5.5	1.60	0.08
20	s	0.74	0.31	0.14	4.6	1.43	0.12
	u	0.73	0.29	0.13	3.9	1.53	0.08
21	s	0.86	0.40	0.18	3.6	1.98	0.15
	u	0.86	0.40	0.18	4.4	2.20	0.10
22	s	0.67	0.31	0.15	3.9	1.70	0.25
	u	0.68	0.32	0.15	5.3	2.08	0.19
$\bar{x} \pm \sigma_s$		0.86 ± 0.04	0.39 ± 0.02	0.19 ± 0.01	6.4 ± 0.5	2.20 ± 0.13	0.23 ± 0.02
$\bar{x} \pm \sigma_u$		0.86 ± 0.04	0.40 ± 0.02	0.19 ± 0.01	7.1 ± 0.5	2.51 ± 0.14	0.17 ± 0.02

Table 3

Summarized data of stimulated (s) and unstimulated (u) muscles treated with glycerol. Weight of fresh muscles (G_f); weight of muscles after glycerol treatment (G_g); water loss due to glycerol treatment in first approximation (W) (for detailed calculation see Hummel, 1980)

		G_f [g]	G_g [g]	W [g]	K [mg]	diff		Na [mg]	diff.	
						[mg]	[meq]		[mg]	[meq]
m.	s	17.94	6.21	~12	43.36	7.09	0.18	6.90	2.07	0.09
sart.	u	17.94	6.21	~12	50.45			4.83		
m.	s	18.92	8.58	~10	48.40	6.82	0.17	5.06	1.32	0.06
semim.	u	18.92	8.80	~10	55.22			3.74		

Discussion

Many investigators (Mitchell et al., 1922; Mond, Netter, 1930; Fenn, 1937; Steinbach, 1947) have found by different methods, that active muscle loses potassium. In choosing our method of investigation the main point of view was to decide: what is the state of potassium in muscle like: 1) is K evenly distributed in cell water, or, 2) the bulk of potassium is bound, i.e. it is in an osmotically inactive state in a certain locus (in the A band of the fibril).

The fact that, after glycerol treatment, more Na remained in the stimulated muscles than in their unstimulated pairs shows that our experimental results are not a consequence of a simple damage to membrane permeability.

In Case 2, the results can easily be interpreted since, according to it, the difference in K loss of stimulated and resting muscles only depends on the quantity of bound potassium released during excitation. But, up to now, this bound state has not yet been clearified. We suppose that a large portion of muscle potassium forms so-called K biocomplexes with organic molecules and bound water molecules. They are not of high stability, they can be destroyed and rebuilt easily, so the excitable cell can respond to any, even subtle, external effect. Ernst found the pre-contractile periodic volume decrease to be closely related to the action current of muscle (Ernst, 1925). This fact is an argument for the existence of K biocomplex, in which the potassium is in dehydrated state and will be released and hydrated during the action current (Ernst, 1963).

Arguments in favour of organic alkali complexes can be found in the dispute between Schlenk (1914) and Ziegler (1930). The handbooks by Sidgwick (1927), Schlenk and Bergmann (1932), Schmidt (1934) furnish further evidence. Nowadays, there are many evidences of the existence of potassium complexes (e.g. Eichhorn, 1973). These complexes possibly have a function in biological excitation. Potassium may be kept among active (charged/polar/polarizable) sides of organic molecule(s). This organic complex will decay under the influence of its activation energy. All in all, the decaying of a K-biocomplex

means a decrease of entropy, because of the entropy decrease of "free" water molecules present in its surroundings, as the relatively free water becomes bound to the opened active sides of the organic molecule(s) and to the released free potassium. This entropy decrease means a "heat quantum" which excites modes of vibration along macromolecule(s), or in the ordered structure of any molecule. Absorbtion of these phonons in the neighbouring K biocomplexes may induce them to decay. In this way the biological excitation can travel by phonon transport *via* K biocomplexes.

On the one hand, facts have been gathering against the view that living cell is an osmotic sac and, on the other, in favour of bound water and bound alkalis. The existence of bound inorganic materials leads to the view of K biocomplexes, which are labile enough to decay during excitation.

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Activity Evoked in Hand Muscles by the Stretch Reflex of m. biceps in Man*

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Experiments were performed on six volunteers, in order to investigate the effect of the activity of the m. biceps branchii on the median and ulnar nerves. The reflex of the m. biceps brachii was evoked by a sudden stretch and the EMG responses of the m. biceps brachii, m. abductor dig. V. and thenar muscles were recorded. Hand muscles produced early EMG potentials, the latency between them, as well as the onset of the electrical response recorded from the m. biceps brachii, being about the same as the conduction time of nerve excitation from biceps to the hand muscles. The possible mechanisms of the origin of the early EMG activity recorded from the hand muscles are discussed.

Introduction

The problem of electrical interaction between excitable tissues is of great importance for a more complete understanding of the motor control of human and animal movements. The fine movements of the distal link in the kinematical chain of a limb is accomplished by stabilizing the position of the proximal links of this chain by means of the activity of the proximal muscles (Bernstein, 1947, 1966).

The nerves innervating the distal muscles of a limb are, in many cases, passing along the proximal muscles and have contact with these muscle fibres through connective tissue.

The excitatory effect of muscle action potential on a nerve being in contact with the muscle was demonstrated by Matteucci in 1842. Later, it was shown that the muscle action potential should excite the motor nerve fibres in the area of their endings within the muscle (Masland, Wigton, 1940; Lloyd, 1942; Eccles et al., 1942; Epstein, Jackson, 1970), and that it had an influence on the activity of the muscle spindles (Granit et al., 1959). Recent publications on the ephaptic responses recorded in man also deserve special attention (Egloff-Baer, Roth, 1979; Roth, Egloff-Baer, 1979).

The nerve excitation generated by muscle action potentials in the neighbourhood of the muscles was demonstrated under *in situ* circumstances (Biró, 1975; Biró, Vu-Duy-Thinh, 1977).

* The experiments were carried out in the Pavlov's Institute of Physiology, Acad. Sci. USSR, Leningrad, USSR.

The results mentioned above allow the hypothesis that the effect of the electrical activity of the muscle exerted on the nerve in contact with it might play an additional role in the regulatory mechanisms which take place in the excitatory processes concerning the nerve and muscle function (Biró, 1975).

However, owing to the lack of experimental data on the analysis of electrical potentials during reflex and voluntary activity under normal condition, no attempt has yet been made to substantiate this hypothesis.

The present work tries to start filling this gap. The stretch reflex of the muscles of proximal extremities seems to be a suitable model for studying this problem. If the proximal muscles have an electrical effect on the nerve, it is to be expected that activity can be generated in the distal muscles which are mechanically independent of the proximal ones.

Materials and methods

The experiments were performed on six healthy male volunteers at the age of 20 to 40.

The m. biceps brachii was chosen as the subject of investigation because the trunks of the median and ulnar nerves are situated under this muscle and the nerves contact with it along a significant length.

The stretch reflex of m. biceps brachii was evoked by means of a special mechanical stimulator (Gerasimenko, Romanov, 1980) delivering a sudden load of 40 N to the middle part of the forearm being in flexion of 90°.* The upper arm postured on a special frame was also at an angle of 90° relative to the body. The hand (in supination) and the forearm were fastened to a splint, in order to immobilize the wrist and the fingers.

Surface electrodes (DISA) were used for recording EMG. The pairs of electrodes were fastened to the skin over the m. abductor dig. V., the group of thenar muscles and the m. biceps brachii. The distances between the electrodes were 2 cm in all cases.

The stretch reflex was evoked about 100 times in each experiment. The responses were followed on the screen of a storage oscilloscope. The temporal relations between the onset points of the EMG recorded from the muscles were measured, and analyzed then by statistical methods.

After the tests of stretch reflex, the conduction velocities of the median and ulnar nerves were determined by means of electrical stimulation of these nerves. The nerve stimulations were applied to the upper arm and the wrist. At the same time, the potentials evoked in the hand muscles were also recorded.

In the last part of the experiments two more tests were performed. The first test was a voluntary contraction of m. biceps brachii, while the second one the tonic vibration reflex. The vibration applied to the tendon of the m. biceps brachii was of a frequency of 80 cps, 1 mm in amplitude and 2 minutes in duration.

* It should be noted that, besides the m. biceps brachii, the stretch affects other muscles, too.

Results

Reflex EMG activity of m. biceps brachii was evoked by sudden pulls given to the forearm of the volunteers in a total of 479 cases. The responses were manifest in the form of a discharge of high amplitude (Fig. 1). The latencies of these responses varied between 38 and 55 ms. The duration of one single response was short because the subjects had received instruction not to resist the pulls and not to try to quickly restore the position of the elbow.

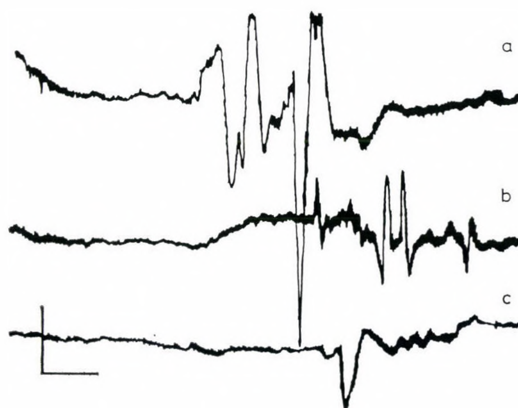

Fig. 1. Evoked potentials of the m. biceps brachii (a), m. abductor dig. V. (b) and thenar muscles (c). The responses were evoked by the stretch reflex of the m. biceps brachii. Calibration: 500 μV for *a* and 100 μV for *b* and *c;* 10 ms for all the beams

As shown in Fig. 1, a certain time after the appearing of the response of the m. biceps brachii, electrical activity could be recorded also from the m. abductor dig. V. and the thenar muscles. However, these responses were not observed in all cases. The average probability of recording the responses evoked by the stretch reflex of m. biceps brachii was 0.76 in the m. abductor dig. V. and 0.52 in the thenar muscles. The data in Table 1 demonstrate that this activity, originated from the hand muscles appears, with high probability (0.90 and 0.94) in some persons and with low one (0.16 and 0.34) in others. The probability of recording these EMG responses did not change during the whole course of the experiments, i.e., this parameter was the same in either the first, middle or last part of the experiments.

Individual latencies between the activity produced by the m. biceps brachii and that of the hand muscles were different. In the case of three subject, the latency was practically identical with the conduction time of nerve excitation from the m. biceps brachii to the hand muscles; in two further persons, however, the latency was two times longer than the conduction time (Table 1). The histograms of the

Table 1

Experimental data obtained from the hand muscles in the case of the stretch reflex of the m. biceps brachii

Subject No.	Probability of EMG responses		Latency of the EMG responses (ms)		Conduction time (ms)	
	m. abd. dig. V.	thenar muscles	m. abd. dig. V.	thenar muscles	m. abd. dig. V.	thenar muscles
1	0.83	0.57	22.5 ± 1.1	26.8 ± 1.6	12	12
2	0.90	0.43	14.4 ± 0.8	14.4 ± 1.1	12	13
3	0.66	0.66	10.5 ± 0.7	7.4 ± 0.5	—	—
4	0.59	0.94	15.8 ± 0.7	13.0 ± 0.6	12	13
5	0.83	0.16	11.5 ± 0.6	12.5 ± 1.1	11	11
6	0.84	0.34	19.5 ± 0.7	20.1 ± 0.9	10	11

latency times are shown in Fig. 2. The distributions reveal two peaks for the m. abductor dig. V. and one peak for the thenar muscles. (The mathematical analysis of the results, interesting as it is, has not been a major aim of the present work.)

After experiments on stretch reflex, instructions were given the subjects to contract the m. biceps brachii with maximal velocity and force. The EMG activity of the m. abductor dig. V. and the thenar muscles appeared in such circumstances, too (Fig. 3). Just like in the case of stretch reflexes, the excitation of the hand muscles was not manifest in every case, although the probability of muscle responses was not less than p = 0.6.

Generally, the activity of the hand muscles precedes the high amplitude EMG potentials recorded from the m. biceps brachii; also spikes could be led from the hand muscles during the high-amplitude activity of m. biceps brachii for a long period of time.

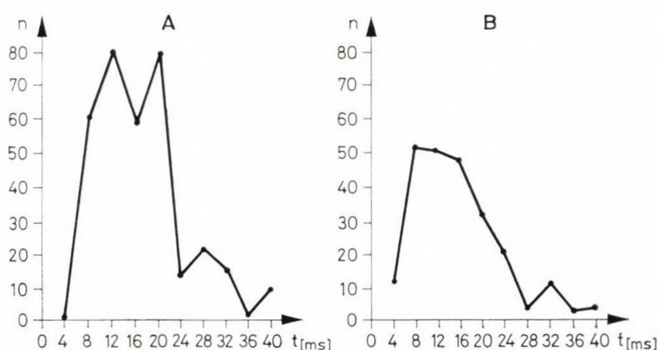

Fig. 2. Histograms of latency times (t) between the onset of electrical activity recorded from the m. biceps brachii and the m. abductor dig. V. (A) as well as the thenar muscles (B)

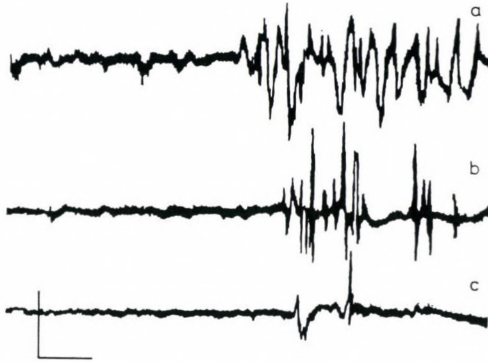

Fig. 3. EMG records from the m. biceps brachii (a), m. abductor dig. V. (b) and the thenar muscle (c) in the case of voluntary contraction of the m. biceps brachii. Calibration: 500 μV for *a* and 100 μV for *b* and *c*; 10 ms for all the beams

The effect of vibration delivered to the tendon of m. biceps brachii elicited the tonic vibration reflex in all the six subjects, but EMG activity of the hand muscles could be recorded in two cases only (Fig. 4). It is characteristic that this EMG activity is superimposed on the action potentials of same amplitude and is generated 20 – 24 sec after the vibration reflex.

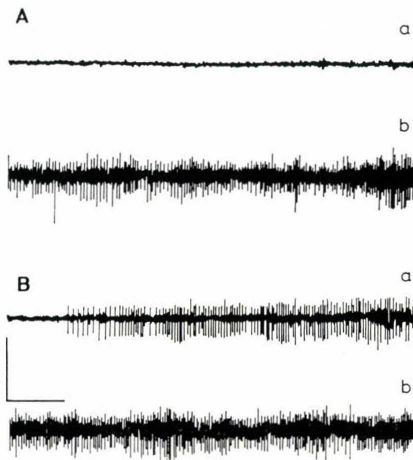

Fig. 4. Electrical activity of m. abductor dig. V. (a) and m. biceps brachii (b) during the tonic vibration reflex of the m. biceps brachii. A: records from 10th sec. B: records from 20th sec. Calibration: 300 μV and 1.5 sec for all the beams

Discussion

One of the possible viewpoints from which the data obtained by studying reflex activity could be analyzed, is the general reaction called non-specific motor facilitation, i.e. the startle reaction. It is known that this reaction can be produced by different unexpected strong-enough stimuli (Landis, Hunt, 1939; Davis, 1948; Larsson, 1956; 1960; Gogan, 1970; Rossignol, 1975). As the stretch was applied to the subjects unexpectedly, this stimulus, which is able to elicit the startle reaction, can be considered as corresponding to the former criterion. In this case, the onset of the activity recorded from the hand muscle could be explained by the non-specific motor facilitation. It is characteristic of the startle reaction that it will disappear after a long train of repeated stimuli of whatever intensity. However, the probability of recording the activity of the hand muscles did not change during the whole course of the experiments.

In addition to the previous argument, it is inferred from the generalized and non-specific nature of this reaction that the motor facilitation is the same for the different muscles. Our results demonstrate that this reaction occurs with individual specificity in different subjects, for the probability of recording the EMG from the two muscles was different. Thus, startle reaction is an insufficient explanation of the observed EMG activity led from the hand muscles.

It is conceivable from another aspect that the facilitatory effect of the diffusely scattered afferent fibres from the muscle spindles, which had been stimulated by the stretch of the m. biceps brachii, on the α-motoneurones and interneurones in the spinal cord resulted in the hand muscle activities (Granit, 1970). Theoretically, it is possible to take this supposition into consideration, because the median nerve innervating the superficial layer of the thenar muscles and the n. musculocutaneus innervating the m. biceps brachii take their origin in almost the same segments (C_{6-7} and C_{5-7}) of the spinal cord. It is a more complicated problem to apply the former supposition to the deep layer of the thenar muscles and of the m. abductor dig. V. innervated by the ulnar nerve (C_8-Th_1'). It is well known that some afferent nerves of the muscle spindles give descending fibres entering the short propriospinal pathway (Kostyuk, 1973) and these nerves can transmit their effect to the lower segments. These polysynaptic connections have higher threshold and need strong and sustained excitatory effect of the stimuli for the activation of the reflex chain. The explanation mentioned above can be supported by the data concerning the fact that during the tonic vibration reflex the activity of the hand muscles appeared in 20–24 sec after the beginning of the EMG response from the m. biceps brachii. At present it has been accepted generally that this reflex is produced by a polysynaptic excitatory effect of the afferent fibres from the muscle spindles on the α-motoneurones (Eklund, Hagbart, 1966; Gillis et al., 1971; Granit, 1970). Therefore, it is not likely that this mechanism plays a role in the reaction taking place rapidly in our experiments.

Considering that the obtained activity of the hand muscle cannot satisfactorily be explained by the mechanisms mentioned above, another alternative

explanation is suggested, according to which the excitatory influence of the action potential generated by the m. biceps brachii or its synergetic muscles accounts for the excitations of median and ulnar nerves producing the observed early EMG activity of the hand muscles. However, it is very difficult to obtain any direct evidence for this kind of explanation in human experiments.

The question of the variancies in the latency time between the activities of the m. biceps brachii and the hand muscles also need more discussion. It is possible that the variability in the latency depends considerably on the condition of the surface recording. These and other questions have to be answered by further experiments.

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A Model System for Bacteriorhodopsin Chromophore

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The absorption characteristics of bacteriorhodopsin chromophore cannot be understand on the basis of a simple protonated Schiff-base linkage. A possible hypothetical explanation may be an interaction of the aromatic amino acid residues also with retinal. Mixtures of retinal and tryptophan analogues were reacted in organic solvents. Many similarities were found in the absorption spectra of the different products of these reactions and in those of the main forms of bacteriorhodopsin photocycle. Such products are suggested to model the purple complex of bacteriorhodopsin as well as the chromophores of the photointermediates.

Introduction

Most authors agree that the principal interaction of the chromophore in visual pigments and BR[2] is a Schiff-base linkage between retinal and a lysine residue (Stoeckenius et al., 1978). Questions remain, however, about the origin of the characteristic red shifted absorption of these chromophores when bound to the protein. Numerous theoretical models have been published in the literature which present a number of very different mechanisms to explain this bathochromic shift (Irving et al., 1970; Honig, 1978; Muthukumar, Weismann, 1978; Kakitani, 1980). Experimentally it has been shown that model Schiff-bases, under rather specific conditions, can reproduce the spectral properties only of visual pigments but not those of the more red-shifted BR (Blatz, Mohler, 1975; Kliger et al., 1977).

A few of the theoretical explanations of the bathochromic shift (Irving et al., 1970; Muthukumar, Weismann, 1978) as well as other observations (Bogomolni et al., 1978) point to the possible existence of close structural and functional interactions between aromatic amino acid residues and the retinylidene moiety. Some scattered papers in the literature deal with the experimental studies of such interactions in model systems (Ishigami et al., 1966; Toth, Rosenberg, 1968; Mendelsohn, 1973; Kliger et al., 1977) but they have not yet led to any generally accepted view.

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[2] Abbrevations used: BR = bacteriorhodopsin; TEA = triethylamine; in P—X: P stays for a product having absorption maximum at X nm.

The present paper describes experimental conditions for systematic investigation of the interactions between retinal and indole derivatives, which model the side chain of tryptophan in organic solvents.

Materials and methods

Diindole-hydrochloride was prepared by bubbling dry HCl through a concentrated $CHCl_3$ solution of indole. The precipitated material was then washed, dried and tested by thin-layer chromatography (Smith, 1963). All experiments were carried out in $CHCl_3$ solutions acidified by bubbling anhydrous HCl or made alkaline by the addition of TEA. The only exception was the reaction of retinal and diindol-HCl, which was processed in neutral ethanol; further studies on the product itself were made in $CHCl_3$. Generally, the concentration of retinal was about 2×10^{-4} mol with indole or diindole-HCl added in high excess.

A combined glass-Ag/AgCl pH electrode pair (Radelkis, Hungary, type OP 8071) was used to measure the pH (see below) of the solutions. The aqueous reference solution in the electrode was exchanged for glacial acetic acid saturated with KCl and AgCl (Mattock, Taylor, 1961).

The electrode potential was measured by means of a Keithly 610 CR electrometer.

$CHCl_3$ was prepared for titration by bubbling anhydrous HCl through the solution until the electrode potential reached saturation level (580 – 610 mV). Then it was titrated by adding either 1 per cent, 10 per cent, or 100 per cent of TEA in $CHCl_3$. Acidic reaction products were titrated by two methods. These two methods differed only in the speed of titration but formed different products. For fast titrations the total amount of TEA was added to the solutions in one step. For slow titrations TEA was added in small increments and the electrode potentials and absorption spectra were allowed to equilibrate. The amount of added TEA was chosen in such a way as to change the electrode potential but by 1 – 2 mV in each step. Since the TEA-HCl salt accumulating during titration exerts undesired effects on the P-415 → P-480 reaction (see below), a part of the HCl has removed by bubbling N_2 through the sample at 40 to 50 °C and replacing the evaporated $CHCl_3$.

Absorption spectra were taken up by means of a Zeiss Specord UV-Vis spectro photo-meter using quartz cells 1 mm in path length and neutral $CHCl_3$ as reference material. All experiments were carried out at room temperature in dim red light.

Results

We found $CHCl_3$ to be the best medium for the synthesis and study of indole and retinal reaction products. In such a solvent the usual pH concept must be modified. We used the potential values measured on the pH electrode pair for defining an empirical function to control and reproduce the acidity–basicity of solvents. This function will be denoted as Φ, which is justified by the observa-

tion of the characteristic S-shaped titration curves found in aqueous media (Fig. 1). In this representation a higher value of potential Φ corresponds to a more acidic solution.

The reaction between indole and retinal at $\Phi = 600$ mV results in the disappearance of retinal absorption at 380 nm and the emergence of a new band at 640 nm. This P-640 product can be converted by fast alkaline titration to a P-490 product ($\Phi = -50$ mV) in a reversible way (Fig. 2a). This observation agrees with the findings of Toth and Rosenberg (1968) as well as of Mendelsohn (1973).

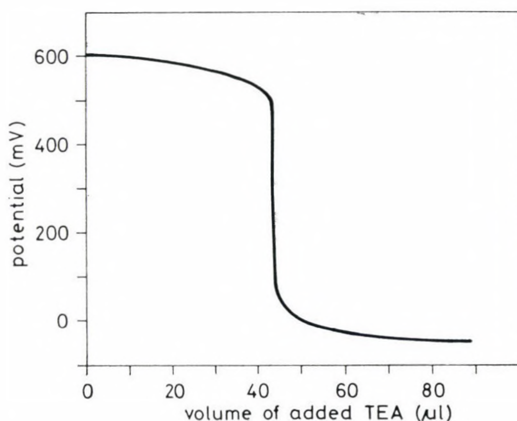

Fig. 1. TEA titration curve of 15 ml $CHCl_3$ acidified with HCl

Interestingly enough, slow titration leads to a quite different result: P-640 is transformed into a P-415 product ($\Phi = 400$ mV) in a partially reversible reaction (Fig. 3). P-415 remains stable in the 400 to 150 mV range and also when Φ is lowered by an additional fast alkaline titration below -50 mV, showing that P-490 and P-415 are produced by different mechanisms. However, if Φ is lowered to the 150 to 0 mV range P-415 will slowly transform into a P-480 product. Acidification of this species leads to the formation of a P-490 product at $\Phi \sim 600$ mV. This last process is also partially reversible in the course of fast Φ changes (Fig. 4a).

The characteristics of the reaction product of diindole-HCl and retinal are similar to those of P-640. In ethanol the absorption of this reaction product is centered at 540 nm and is shifted to 566 nm in $CHCl_3$ (P-566). It is stable in the 300 to 500 mV range; fast alkaline titration ($\Phi = -50$ to -100 mV) can convert P-566 to a P-340 form in a partially reversible way (Fig. 2b). It is known that in acidified aprotic solvents the conjugate acids of the dimers of indole derivatives are always formed as by-products (Smith, 1963); other authors (Ishigami et al., 1966; Toth, Rosenberg, 1968) using similar conditions found products similar to P-566.

It is possible that the blue shift in the absorption of P-566, as compared with P-640, is caused by the saturated double bond between C-2 and C-3 in one

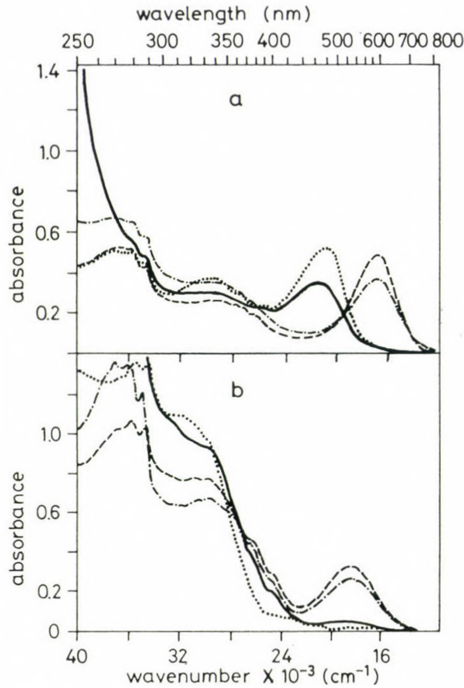

Fig. 2a. (—·—·) P-640 at 580 mV, (——) converted to P-490 by fast titration (−50 mV), (----) converted back to P-640 (580 mV);
b. (----) P-566 at 350 mV, (——) converted to P-340 by fast titration (−90 mV), (—·—·) converted back to P-566 (580 mV)

of the indole rings in diindole. To confirm this hypothesis we make 2,3-dihydro-indole to react with retinal under the conditions described for production of P-640. The resulting product had an absorption spectrum practically identical with that of P-566.

The slow titration reaction of P-566 resembles that of P-640 but consists of one step only. The resulting product (Fig. 4b) has a poorly defined absorption maximum in the 300 to 350 nm range (P-300−350, $\Phi = 0$ to -100 mV). At $\Phi =$ $= 450$ to 550 mV it is converted to a P-545 product; also this reaction is partially reversible on fast Φ changes.

Because the solution was acidified by dry HCl gas bubbling, the speed of acidification could not easily be controlled. It was observed, however, that an alternative path to produce P-590 and P-545 was opened by the slow acidification of P-490 and P-340, respectively.

A cummulative scheme of the above reactions is presented in Fig. 5. It can be seen that products of indole and diindole behave in a similar fashion when titrated. The only exception is the formation of P-415. A general feature is the

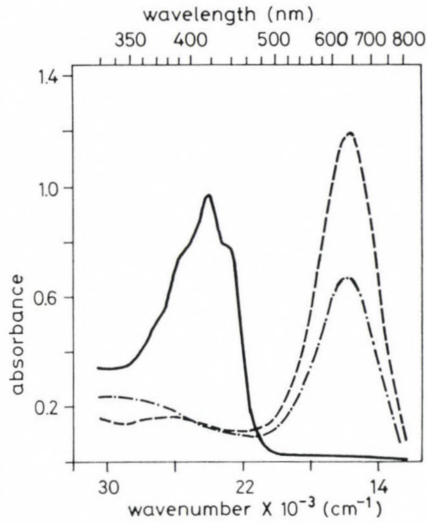

Fig. 3. (----) P-640 at 590 mV, (——) converted to P-415 by slow titration (400 mV), (-·-·) converted back to P-640 (580 mV)

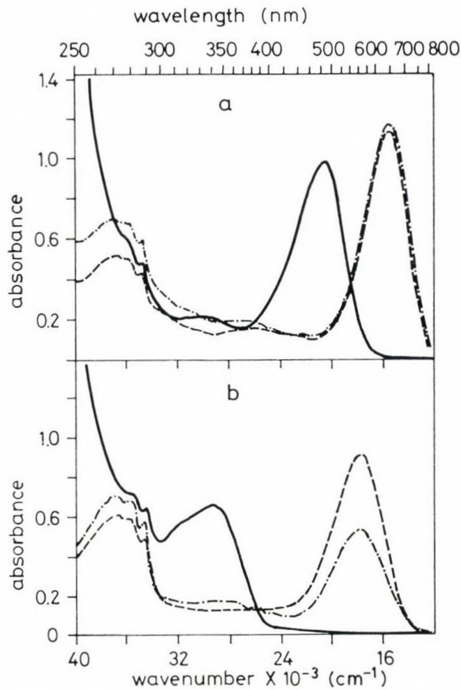

Fig. 4a. (. . . .) P-480 at 80 mV, (----) converted to P-590 (590 mV), (——) converted back to P-480 by fast titration (110 mV), (-.-.) converted again to P-590 (580 mV); b. (. . . .) P-300—350 at 0 mV, (----) converted to P-545 (480 mV), (——) converted back to P-300—350 by fast titration (—80 mV), (-·-·) converted again to P-545 (550 mV)

marked blue and red shift in the absorptions during alkaline and acidic titrations, respectively. A small blue shift takes place in the absorption of all forms produced by slow titration as compared to that of the corresponding fast titrated ones.

Under the conditions described retinyl Schiff-bases and tryptophan analogues generally fail to produce samples with absorption maxima above 500 nm (Kliger et al., 1977). At the same time we observed slow appearence of a purple material when the solution of retinyliden-lysine was condensed to solid in the presence of very high excess of diindole-HCl. It is unknown, however, whether or not the Schiff-base underwent modification under such conditions.

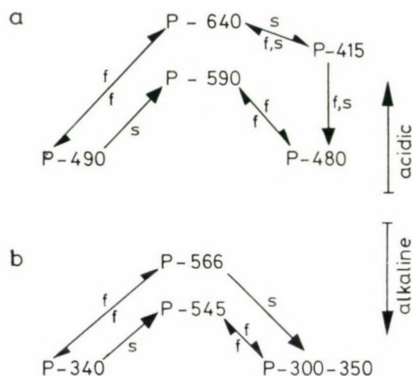

Fig. 5. The scheme of the reaction paths of retinal with (a) indole and (b) diindole. Fast and slow Φ changes are denoted by f and s

Discussion

The most striking feature of P-566, P-590, P-545, P-415 and P-640 is that their absorptions are very similar to those of the BR_{568}, K_{590}, L_{550}, M_{412} and O_{640} forms of BR (Stoeckenius et al., 1978). In addition, the structured spectrum of P-415 and its existence in non-acidic environment also resembles the characteristics of M_{412} (Schreckenbach et al., 1977; Stoeckenius et al., 1978). On the basis of these analogues the above products may serve as models for the corresponding BR forms. [See also Marcus and Lewis (1978) for P-480 and P-490.]

An obvious explanation of the bathochromic shift in the reaction products is that an electronic linkage takes place between the π electron system of the retinal chain and the indole ring. The shifts would then simply be caused by the extension of electron delocalization *via* this linkage. This is in accordance with the difference in the absorption maximum of P-640 and P-566, the latter being more saturated; so, the delocalization is more restricted. The charge of the proton also contributes to the red shift, as expressed by the marked blue shift upon deprotonation. Both the electronic linkage (Bogomolni et al., 1978) and charge

perturbation (Honig, 1978) are also regarded as essential in the determination of the spectral features of the BR chromophore.

The actual nature of the interaction between retinal and indole derivatives cannot be determined on the basis of the present data. The findings of other authors (Ishigami et al., 1966; Toth, Rosenberg, 1968; Mendelsohn, 1973) concerning this interaction are contradictory.

Secondary interactions, for example charge transfer interactions of H bonds, are more consistent with the Schiff-base model of the BR chromophore than are covalent bonds. In this case the aldehyde group of retinal does not play any essential role, so it can probably be replaced by a Schiff-base. At this time, we cannot rule out the existence of a covalent bond. It is also possible that, in addition to a covalent bond, another secondary interaction is responsible for the charge distribution resulting in an absorption similar to that of the BR forms.

For construction of an actual model of BR chromophore on the basis of the present findings further purification and the determination of the exact structure of the described products are still needed.

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Dose Reduction in the Low-energy X-ray Diagnostics of Extremities

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The authors determined the surface absorbed dose (D_s = 67–68 mGy) and the average absorbed dose (D_{av} = 13–15 mGy) by means of non-screen film radiography of the extremities. The authors recommend the use of xeroradiography (with a tungsten anode tube) or, first of all, a rare-earth screen — film combination (e. g. Kodak min-R + Nuklearmedizin NMB film). The two methods permit the dose values to be decreased by a factor of about 4 and 15, respectively. The dose values are smaller, at least for the 8 cm thick tissue equivalent phantom, when a tungsten anode is used instead of a Mo anode.

Introduction

The native X-ray examination of the soft tissues of extremities has not yet been widely accepted. We have so far examined more than a thousand patients and, with this non-invasive procedure, obtained new *in vivo* information which could not otherwise be obtained, except by means of operation at obduction.

From a diagnostic point of view the method has proved most valuable in traumatology for exploring injuries of the muscles and tendons of the extremities, when soft tissue radiographs provide an objective picture of the traumatic morphological changes (Pálvölgyi, 1978a; Pálvölgyi, Bálint, 1979). When the motoneurons are injured both the degree and nature of the damage to the individual muscles can be determined. It has been demonstrated that, during neuromuscular and inflammatory diseases, certain muscles or parts of muscles undergo damages of different extent. New data have been obtained with respect to the pseudohypertrophy and atrophy of the musculature and to the pathological changes adherent to elderly (Pálvölgyi, 1978b; Pálvölgyi, Gallai, 1980; Pálvölgyi et al., 1977).

It has been the purpose of this paper to measure the absorbed dose in X-ray diagnostics of soft tissues and to find other methods which would decrease the patient's exposure.

Materials and methods

Routine patient diagnostics

The technique developed for X-ray examination of soft tissue and the diagnostical use of this method have been described by Betsch et al. (1966).

The most current examinations are those of the calf musculature with mediolateral X-ray beam (the tibia is not exposed). Sagittal X rays of the calf are taken in exceptional cases only.

X rays of the thighs are taken relatively rarely. Diagnostically valuable roentgenograms can only be taken of the distal half and mainly in thin patients. X rays of the soft tissue of the lower arm were taken in supine position with a beam penetrating from the surface of the palm.

The vast majority of the radiographs had been taken on Agfa-Gevaert Structurix D_{7p} non-screen film without cassette, using standard equipment (with 6 valve tubes) and a W anode tube with a focal spot of 2 mm. For calf musculature of average size (about 8 cm) the following exposure settings were used: 30 kV; 200 mA; 3.2 sec. The focus skin distance (FSD) was about 42 cm.

The examination of the upper extremities was performed with reduced exposure time (1.6 – 2.0 sec).

X-ray sources and imaging systems

In the present measurements three X-ray generators were used. The diagnostic examinations of the patients were made in the National Institute for Nervous and Mental Diseases, by means of an EDR-750 generator (Medicor, Hungary) with a rotating tungsten anode tube (inherent filtration without any filter was 2 mm Al equivalent focal spot 2 mm) at a focus-film-distance (FFD) of 50 cm. The Agfa-Gevaert Structurix D_{7p} film was developed at 22 °C for 6 min.

The Siemens Gigantos E X-ray unit with a rotating W anode Biangulix tube (B; 150/30/50 mA) was used at the County Hospital, Szekszárd, with the small focal spot (1.2 mm). The inherent filtration of the X-ray tube was only 1 mm Al, as 1 mm Al was removed from the exit port. The FFD was 70 cm. The Mo anode unit of the latter hospital is a Senographe. It was used at a FFD of 50 cm with 0.5 mm Al filtration.

At the Szekszárd County Hospital various receptors were used. The Agfa-Gevaert Structurix D_{7p} films were developed at 22 °C for 6 min. The 10-dose system consisted of a Kodak cassette with min-R screen combined with Kodak Nuklearmedizin NMB film which was processed in a 3M processor at 28 °C. Xeroradiography was made by using a Rank Xerox S 125 System in negative mode, contrast setting "D", density level "C". The plate back bias was 3050 V.

Other parameters (high voltage, mAs) of the various source-detector combinations are summarized in Table 1. Comparison of the different techniques was made in Szekszárd. The values listed in the Table were determined on the same amputated lower leg and are the optima of various kV and mAs combinations. The thickness was 8 cm, not too much different from that of an average leg.

Determination of absorbed dose

The phantom material used in our previous investigation (Zaránd, Péntek, 1980) could not be used in the present measurements, since the absorption and attenuation properties of the female breast differ from those of the muscles of the extremities. Therefore, we used tissue equivalent bricks ($4 \times 14 \times 42$ mm^3, manufactured by RFT, GDR) to build a $10 \times 10 \times 8$ cm^3 phantom. The depth dose was measured with ionization method in the central axis. Since our ionization chamber

Table 1

Average absorbed dose (D_{av}) and surface absorbed dose (D_s) in various low-energy radiography techniques of the extremities (phantom thickness: 8 cm)

High voltage, kV	Filtration, mm	Anode	FSD, cm	mAs	Receptor	D_s	D_{av}	$\frac{D_{low}}{D_{av}}$
						mGy per picture		
30	1 Al	W[a]	62	1500	film[d]	67.8	14.7	0.16
35	1 Al	W	62	102	min-R[e]	4.6	0.99	0.20
30	1 Al	W	62	250	XR[f]	16.0	4.0	0.16
30	0.5 Al	Mo[b]	42	165	min-R	22.6	3.6	0.82
45	0.5 Al	Mo	42	210	XR	103	18.9	0.65
30[g]	2.0 Al	W[c]	42	640	film	66.8	13.4	0.28

[a] Siemens Gigantos E
[b] Senographe CGR
[c] EDR-750 Medicor
[d] Agfa-Gevaert Structurix D_{7p}
[e] Kodak min-R screen + Nuklearmedizin NMB film
[f] Rank Xerox System 125
[g] Patience data

had been calibrated in exposure, the absorbed dose was calculated by converting the exposure to absorbed dose in muscle. The mass energy absorption coefficients were calculated on the base of ICRU Report 17 (ICRU, 1970), and were found to be satisfactory constant in the energy range considered (cGy/R = 0.915 ± ± 0.01). The real depth dose curves obtained with this method were integrated and averaged along the depth of the phantom (D_{av}). Each depth dose curve, fitted to the measured points, is within the limits of error sum of two exponentials. The surface intensities of the two components and the linear attenuation coefficients are considerably different depending, first of all, on the anode material and on the potential.

Results and discussion

The results of our measurements are summarized in the last columns of Table 1. D_{low} is the fraction of average absorbed dose due to the "low-energy" photons of the X-ray spectrum. The linear attenuation coefficient of this part suggests that the average energy is well below 10 keV, when a tungsten anode is used. In the case of a Mo anode tube the low-energy part is characterized by the K_α and K_β lines of the anode. It is clear from the analysis of the depth dose curves that the low-energy part of the spectrum is practically absorbed in the phantom and its contribution to the imaging is negligible, in spite of its importance in the absorbed dose. The molibdenum anode tubes should, therefore, be avoided in most of the low-voltage examinations of the extremities. Our results are in good agreement with the measurements of Panzer et al. (Panzer et al., 1978). These authors measured the X-ray spectrum of a Mo anode tube (exciting potential

25 – 50 kV, 0.03 Mo or 0.5 mm Al filtration) behind a Mix-D phantom 0 to 8 cm in thickness. The K lines of the anode were only small spikes on the continuous spectrum when an 0.5 mm Al filter was used and the Mix-D was 8 cm thick. The role of the characteristic X rays is more pronounced when an 0.03 mm Mo filter is applied or when the tube potential is decreased.

The most important result of the intercomparison is that the 10-dose system permits a dose reduction (relative to the film) by a factor of about 15, while the absorbed dose per xeroradiogram is about a quarter of that of the film for a tungsten anode tube. The dose/picture is less whenever a W anode tube is used instead of a Mo anode unit. The Mo anode xeroradiography is not feasible because of the very high dose due to the Mo K lines. We can expect on the base of previous investigations on "high filtration xeromammography" (Techn. Appl. Bull., 1976) combined with the D_{low}/D_{av} ratios of Table 1 that the absorbed dose per picture may be reduced by additional filtration without considerable loss in image quality.

Conclusion

The authors have determined the absorbed dose per picture in low-energy radiography of the extremities. The average absorbed dose per picture may be reduced by a factor of about 15 when a 10-dose system (Kodak min-R Nuklearmedizin NMB film) is used instead of a Structurix D_{7p} film. The dose reduction is less (only by a factor of 3.7) when xeroradiography is used. The low-energy radiography of the extremities is a valuable diagnostic method, and not even the high surface absorbed dose (67 mGy per picture) and average absorbed dose (13.4 mGy per picture) mean contraindication of a conventional film technique if the other methods are not available.

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Ultrafast Charge Separation in Purple Membrane

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The appearance of different intermediates of bacteriorhodopsin photocycle is accompanied by electric pulses connected to the proton pump of purple membrane. Until now these signals were studied by using measuring systems with a resolving time larger than 0.1 μs. It is known, however, that the first intermediates of the cycle are developed during a few picoseconds. One can expect that an early charge separation takes place in the same time intervals. The aim of this work was to demonstrate the existence of electric pulse(s) belonging to such fast charge movement(s).

In the course of the experiments, dried and oriented purple membrane samples were excited with picosecond laser pulses and the electric responses followed on fast oscilloscopes. In this way we succeeded in demonstrating the existence of a new signal which precedes the known ones. The rise time of this signal is shorter than 800 picosecond.

Movement of Protein Side Chains in Bacteriorhodopsin

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During the photocycle of the bacteriorhodopsin linear dichroism has been measured. The anisotropy of the samples was produced by the photoselection method. Measurements were performed at room temperature on purple membrane fragments embedded in agar-agar gel with 100 μs time resolution at several wave lengths in the spectral region of 240 – 550 nm.

The experimental data prove that neither the retinal nor the aromatic protein side chains which participate in the proton pumping function show any rotational movements correlated with the photocycle.

Temperature Dependence of Displacement Currents Measured on Oriented Suspensions of Purple Membranes

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On oriented purple membrane suspensions displacement current signals can be measured following flash excitation. We have previously shown that this signal is a sum of exponentials, the exponents being equal to the rates of successive reactions in the photocycle of bacteriorhodopsin.

We have succeded in separating the electric signal which belongs to the first, i.e. BR $\rightarrow$ K transition.

In order to obtain further proof that the displacement current originates from known steps of the photocycle we investigated the temperature dependence of the signal. The activation energies of the steps were found to equal those of the thermal reactions in the photocycle when determined by absorption-kinetic measurements. It has been shown that the signal originates from movement of protons during the photocycle; this is supported by the temperature dependence of the amplitudes of the current signal.

Electro-optical Measurements on Aqueous Suspension of Purple Membrane Fragments

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Purple membrane fragments from *Halobacterium halobium* can be ordered in electric field. Static electric field orients the sample mainly due to the permanent dipole moment, which is perpendicular to the membrane surface, while the alternating electric field alignes the membrane fragments due to the induced dipole moment along the surface. Electric dichroism measurements on these samples give a possibility to determine the value of permanent and induced dipole moment as well as the angle of the retinal to the membrane plane normal. These values were calculated by varying the pH, also taking into account the pH-dependent

aggregation of membrane fragments. Both the permanent and the induced dipole moments have a maximum at about pH 6 and pH 5, respectively. At pH > 5 the negative charge of the permanent dipole moment coincides with the internal while, in case of pH < 5, with the external side of the membrane.

Dried Oriented Purple Membrane Samples

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Stable, oriented samples from purple membrane of *Halobacterium halobium* were prepared, in order to study their electrical and optical properties. The permanent dipole moment of the membranes was used to orient, and the net negative charge to move them electrophoretically to the surface of the anode. The orientation was stabilized by drying the samples. The samples were very stable for long time (for months) and produced large photoelectric signals (about 10 V under continuous light). Studying the dependence of the photoelectric signal on humidity in continuous light we found that increased dehydration level decreased light adaptation of the bacteriorhodopsin and that about 10 per cent of the relative humidity disappeared.

The time constants of the electric and optic photoresponse signals to flash excitation correlated with the time constants of the bacteriorhodopsin photocycle.

Proton Movement in Mitochondria

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Our studies were carried out on rat liver mitochondria and submitochondrial particles. A new measuring system was developed for the experiments, in order to get information on several materials having influences on the mitochondrial proton transport.

1. Diethylpyrocarbonate (DEP): at about pH 6.5 it has a specificity for histidine blocking at the outer part of the inner mitochondrial membranes. 10 μmol DEP/1 mg mitochondrial protein shows a complete blockade, lower concentrations of DEP have no effect.

2. Potassium ferricyanide: it has a higher affinity to cytochrome *c* than has cytochrome *c* to cytochrome oxidase. After the reduction of ferricyanide the medium becomes more acidic but this pH difference does not produce ATP.

3. Hydroxylamine: it causes a partial (light sensitive) blocking of the O_2 uptake.

Some Aspects of Fluorescence Polarization of Chloroplasts Oriented in Magnetic Field

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The effect of magnetic field on the orientation of phostosynthetic units was first observed by Geacintov et al. The method can be used for investigation of the organization of pigments in photosynthetic membranes. The following results are presented:

The angular dependence of fluorescence polarization in saturated magnetic field.

The parallel ($F_{||}$) and the perpendicular ($F_{\perp}$) components of the fluorescence radiation depend on the chloroplast concentration in a characteristic way: $F_{||}$ increases, while $F_{\perp}$ decreases with concentration. This is an orientation effect.

The fluorescence ratio $F_{\perp}/F_{||}$ decreases with increasing temperature: at around 20 °C a change can be observed, which is related to the phase-transition in thylakoid membrane.

The experimental results are compared with a simple theoretical model calculation.

Aggregation of Protochlorophyll in Solid Film Model System

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A great number of results derived from spectroscopic measurements shows that the protochlorophyll(ide) (Pchl(ide)) – similarly to other photosynthetic pigments – has different forms *in vivo*. These forms are characterized in the literature by their red absorption and/or fluorescence maxima. For studying the molecular background of the differences in the spectroscopic properties of the pigment forms model systems of different nature are often used. In our work solid films of Pchl were prepared on cover glass surface from diethyl ether solution of the Pchl by evaporation of the solvent. The prepared films were treated with water vapour at 40 °C *in vacuo*. Depending on the length of this treatment a red shift of various degree was observed in the absorption spectra of the films: at first, the main absorption maximum shifter from 635 nm (the absorption maximum

before treatment) to 640, then to 652 nm (and also a shoulder appeared at about 680 nm). The treated films had large and very characteristic circular dichroism (CD) signals, and the CD spectra proved to be very sensitive to the arising of the Pchl aggregates: the sign and the position of the CD signals showed great changes. During the treatment several Pchl films with absorption maximum at 652 nm but with different CD signals were observed. These differences can be caused by the overlapping of several Cotton effects of CD spectra of different forms existing together in the same film, but a more considerable factor can probably be the differences in the geometry of the Pchl aggregates. Our results show that for exact characterization of a pigment form is not enough to indicate the absorption (or fluorescence) maxima, because aggregates with different geometry may have very similar absorption (or fluorescence) spectra. In our opinion, these aggregates cannot be taken as one and the same form.

Investigation of Chlorophyll Fluorescence in Plants Treated with Photosynthesis Inhibiting Benzonitriles

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Five-day-old etiolated wheat seedlings were treated through the roots with 3×10^{-5} mole dm^{-3} of different benzonitriles in darkness for 24 hours. The seedlings were then irradiated with a "white light" of 27 W m^{-2} (5000 lux) and kept continuously in the herbicide solution.

3-Nitro-5-bromo-4-hydroxy-benzonitrile caused a 9–10 nm shift toward shorter wave lengths and increased the half band-width in the fluorescence maximum of the wheat leaves [measurement at long wave lengths at temperature of liquid nitrogen].

There was no similar effect in the case of either 3,5-dibromo- or 3,5-dinitro--4-hydroxy-benzonitrile. The differing effectiveness of these sterically similar molecules is based on their different absorption and translocation processes influenced by their different electron configuration patterns. Fluorescence induction of giant internodal cells of *Nitellopsis obtusa* (Characeae) was investigated under the influence of benzonitriles (6×10^{-5} mole dm^{-3}). The most effective compound, when calculated on the basis of the basis of the F_v/F_{max} values, was 4-nitro-5-iodo-4-hydroxy-benzonitrile. The characteristics of the fluorescence induction curves are similar to those of leaves treated with DCMU. This indicates that the inhibition of photosynthetic electron transport chain takes place on the reducing side of PS II.

Kinetics of the 515 nm Electrochromic Absorbance Change and Configuration of the Electric Field in Photosynthetic Membranes

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The complex kinetics of the flash-induced absorbance change in chloroplasts, ΔA_{515}, between about 1 ms and 500 ms are interpreted using a kinetic model. Our interpretation connects the slow rise with the rate-limited charge-transport following the primary charge separation using the assumption that, upon the translocation of charges through the boundaries of the membrane, the corresponding electric field changes considerably. A fast decaying component is explained by a rapid discharge of a certain portion of membrane population above a critical field strength. The model describes the effect of different experimental conditions on the kinetics of ΔA_{515}. Theoretical calculations carried out in a model of the thylakoid yielded typical configurations and intensities of the electric field induced by charges either localized in the membrane or delocalized in the conductive phases bordering the membrane. This supports our assumption that the slow rise of ΔA_{515} can be related to the rate-limiting electron-transport processes.

Hydrogen Production by Bacterial Extracts

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The phototrophic purple sulphur bacterium *Thiocapsa roseopersicina* contains a hydrogenase enzyme remarkably stable against oxygen and heat. The membrane-free extract of this bacterium, prepared by sonication and ultracentrifugation, forms a system that produces molecular hydrogen. The electron donors of the H_2 producing reaction are the photosynthetic organic materials synthetized by the bacteria themselves. The hydrogen production shows a pronounced increase when glucose is added to the system. An energy conversion efficiency of about 8 per cent has been measured for glucose which is several times higher than the efficiency of similar biological hydrogen generating systems and, therefore, it may be of interest also from a practical point of view. Beside the introduction of the new H_2 producing system experimental data regarding the possible mechanism of hydrogen production and optimalization will be presented.

Localization of Hydrogenase in the Photosynthetic Membrane of *Thiocapsa roseopersicina*

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Thiocapsa roseopersicina, a purple sulphur phototrophic bacterium displays reversible hydrogenase activity *in vivo*, i.e. it catalyzes the reaction:

$$2\,e^- + 2\,H^+ \rightleftharpoons H_2$$

In the presence of artificial electron donors [methyl-(MV^+)- or benzyl-viologen (BV^+) reduced by dithionite] the cells produce molecular hydrogen, while H_2 consumption takes place in the presence of H_2 and oxidized viologens. Protoplasts also show hydrogenase activity of the same magnitude. When protoplasts are disrupted by osmotic shock the activity is found in the sediments of the membrane fraction; therefore, the enzyme is considered to be membrane-bound.

The membrane of the bacterium is impermeable for oxidized redox dyes (MV^{2+}, BV^{2+}) while the reduced forms (MV^+, BV^+) easily penetrate the cells. Exploiting this permeability difference the location of the hydrogenase within the membrane has been determined from H_2 uptake measurements. The results confirm that the enzyme is plasma membrane bound, and reveal that its active centre is at the outer surface of the plasma membrane.

The Microsecond Delayed Fluorescence of the Second Photochemical System of Photosynthesis

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The delayed fluorescence of dark adapted, intact and chemically treated *Chlorella algae* was measured at room temperature with a nitrogen laser phosphoroscope in the microsecond time range using photon counting technique and electronically gated photomultiplier. The maximum time resolution and the dead time of the equipment were 4 μs. For chemical treatment 3-(3,4-dichlorophenyl)-1,1-dimethylurea and NH_2OH were used. The multiphasic decay of the delayed fluorescence could be explained by Delrieu's parallel, heterogeneous reaction centre model of the second photochemical system of photosynthesis.

Membrane Potential Dependent Calcium Transients and Birefrigence Signals in Skeletal Muscle Fibres

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The birefrigence signals and Ca^{2+} transients evoked by depolarizing pulses were studied simultaneously in voltage clamped cut muscle fibres of the frog *Rana esculenta* (T = 2 – 4 °C). Changes in birefrigence were measured with crossed polarizers at 790 nm wave length. Using the metallochromic indicator dye antipyrylazo III intracellular Ca^{2+} transients were recorded at 720 nm. The birefrigence signals had longer latency times, reached their peak value later and decreased slower than the corresponding Ca^{2+} transients evoked by depolarizing pulses of the same amplitude and duration. The peak values of the signals showed the same voltage dependence in both cases. The results suggest that birefrigence transients do not reflect the changes in the membrane potantial of the sarcoplasmic reticulum but rather follow the changes in the intracellular Ca^{2+} concentration. In most cases the falling phase of the birefrigence signals has a much slower time course than the change in the myoplasmic free Ca^{2+} indicating, thus, the role of other processes in eliciting the birefrigence changes.

Shortening of A Bands in Mammalian Heart Muscle

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Many morphological investigations proved that, even in case of very different muscle lengths the length of the A band did not change perceptibly either during stretch or during isotonic or isometric contraction. Changes in the length of the sarcomere are accounted for by changes in the length of the I bands alone. On the other hand a severe ischemic injury followed by reperfusion gave rise to the development of supercontracted bands in the papillary muscle of mammalian heart. The striated pattern of the muscle could hardly be recognized in the supercontracted bands any more, and the contractile proteins aggregated irreversibly.

It seems very probable that also the myofilaments themselves could become shorter under similar circumstances. This phenomenon could be demonstrated by the effect of slight ischemic injury, applied after hypothermic potassium cardioplegia plus reperfusion. In this case two distinct types of muscle shortening could be observed:

1. In the intact fibres the shortening of myofibrils corresponded to the sliding filament model. In supercontracted state contraction bands were formed around the Z lines.

2. In some fibres damaged by slight ischemic effect, where the ischemic injury mainly restricted to the sarcolemma and caused redistribution of the diffusible elements, the muscle shortening in supercontracted state was manifest in a real A band shortening only. The sarcomeres became thicker and the length of A bands shortened to the half without any contraction bands around the Z lines.

A single myofibril could contain both shorter and normal A bands facing one another.

This findings prove that myosin filaments are capable of shortening during muscle contraction but, of course, this shortening may be an irreversible process.

The Structural Basis of "Stretch Activation" in the Insect Flight Muscle

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In the Ca^{2+} activated state of the insect flight muscle small applied length changes result in large changes both in maintained tension and maintained ATPase activity. This phenomenon of muscle activation is termed "stretch activation". C filaments are supposed to play an important role in stretch activation of fibrillar muscle. In order to support this hypothesis the following experiments were performed:

The glycerol-extracted flight muscle of bee *(Apis mellifica)* was stretched in rigor solution. Since the sliding movement of the filaments had been inhibited stretch broke away the actin filaments from one of the Z lines asymetrically in some sarcomeres and only the C filaments strained the myosin filaments in the broken half sarcomere. After the stretch the broken actin filaments remained in their original position. When the muscle was transferred to activated solution the developed tension was as high as to force the broken actin filaments to move through the M line producing a wide double overlap zone in the intact half sarcomere. Very often the broken actin filaments slided through the M line completely from one half of the sarcomere into the other in such a way that the intact half sarcomere became a total double overlap zone, while in the broken half sarcomere the half A band became similar to an H-zone. But when the muscle stretched in rigor was released and then treated with activated solution the broken actin filaments maintained their original position, or their movement toward the M line was strongly limited producing, thus, very narrow double overlap zone.

The findings of our experiments support the idea that the strain of the myosin filaments caused by the C filaments results in stretch activation.

Role of Redox State Potential in the Regulation of Muscle Activity

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The trend of changes of redox state potential in muscles following indirect electrical stimulation *in vivo* depends on the frequency of stimulation, i.e. slow stimulation rate evokes an acute redosis, while a quick frequency results in an oxidosis. The acute redox state potential changes brought about in the early phase of stimulation will then be followed by compensatory ones (acute oxidosis after a high, long-lasting redosis and *vice versa*). If the acute redox changes are permanently repeating themselves the compensatory redox changes will exhibit an accumulating character and basic redox change potential will be gradually shifted. On the basis of these processes the following hypothesis might be put forward concerning the differentiation of embryonal muscle tissues: Embryonal muscle → slow stimulus acute redosis → compensatory oxidosis → increased catabolism → red muscle.

Investigation on the Relationship between Stimulus-threshold and Water Content of the Muscle

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The experiments were performed in a closed system containing muscle and distilled water. The water content of the muscle and the vapour pressure of the distilled water could be varied. Water in the muscle was gradually decreased. There was a quasi-equilibrium between vapour pressure of the muscle water and that of the cooler distilled water during drying. This method enabled all fibres to desiccate to an equal extent.

Muscle was gradually dried and the stimulus threshold measured.

After a decrease of about 30 per cent in muscle water content, the muscle could not be stimulated any more.

Effect of Bivalent Cations on Muscle Membrane

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The membrane-stabilizing and capacity-increasing effects of Zn^{2+} ions were studied by analyzing the current-voltage characteristics measured in bilayer lipid membranes (BLM) prepared from oxidized cholesterol.

The hyperpolarizing current voltage characteristics of frog sartorius muscle fibres were measured with a similar method, and it was found that Zn^{2+} ions increased the "saturation current" of the membrane. In the present experiment a square pulse electrotonic analysis was used for determining the electric parameters of muscle fibre. It was examined how Zn^{2+}, Mn^{2+}, Mg^{2+} and procaine cations would modify the time constant, space constant, capacity and transverse resistance of muscle membrane in comparison with untreated muscles.

The time constant, space constant and transverse resistance of muscle fibre were found to significantly increase ($p = 0.01$) upon the effect of Zn^{2+} ions applied in a concentration of $2.5 \cdot 10^{-4}$ M/l. When compared with control values no significant change was found with the other cation investigated.

Role of Ca in Function of the Heart

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In our previous work we investigated the role of Ca in muscle function by electron microscopic autoradiography, then we continued the investigations with heart muscles working automatically. We have studied the localization of Ca in ventricle and auricle of the heart muscle and also the localization of Ca in sinus nodes ensuring the automatism of the heart.

Comparison of different parts of the functioning heart muscles revealed that ventricles and auricles incorporated ^{45}Ca to a significantly greater extent than did the sinus nodes.

The results seem to support the view that more incorporated Ca can be found in the muscle of the functioning heart than in the different kinds of cells.

Energy Demand of Pre-etching of Skeletal Muscles and its Effect on the Contraction Parameters

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We have measured the glucose consumption, lactic acid production and oxygen uptake of M. triceps surae of the hind limbs in the rabbit. The observations have been carried out in both resting and pre-stretched states of different degree over a definite time. Further, we have investigated the effect of pre-stretching on the stimulus threshold, contraction strength, half-times of contraction and relaxation as well as on the degree and rate of the shortening. Relationships could be

found between the tetanic stimulus threshold and fusion frequency and also between the tetanic stimulus threshold and degree of pre-stretching.

It has been shown by our earlier investigations that the atrophy of the skeletal muscles due to inactivation (immobilization with plaster-bandage, hypokinesis) can be prevented by bringing the skeletal muscle to a rest in the stretched state. In the present work we have looked for an answer to the question of whether the changes taking place in the background of this process are or are not primarily trophic (neurogenic or circulatory) in origin. According to our hypothesis, the protective effect of pre-stretching can be explained by changes in the contractile or sarcotubular system and the membrane structures, respectively, which are in connection with the energy balance of the muscle.

Effect of Monovalent Cations on the Solvate Shell of Bioactive Compounds

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The biological effect of a compound depends on its ability to contact the target organism and to penetrate the membrane of the cell. These processes are governed by the adsorptivity and lipophylity of the compound. Recent research indicates that the ion environment considerably modifies the biological effect. This is caused not only by the competition of ions and bioactive compounds for the adsorptive sites but also by the change of the neutralizing solvate shell around the interacting molecules exposing better their inherent characteristics.

We established that the monovalent cations decreased the lipophylity and adsorptivity of hydrophylic molecules and substituents in the order Cs > K > Na. The correlation between the ion concentration and lipophylity or adsorptivity is a semilogarithmic one. In the case of lipophylic molecules and substituents an opposite situation was observed, i.e. the adsorptivity and lipophylity increased in ion environment in the order Na > K > Cs. The type of correlation was the same but the effect much more lower than in the case of hydrophylic compounds. The overall reaction to ion concentration of a molecule containing hydrophylic and lipophylic substituents is chiefly determined by the hydrophylic group. The behaviour of a compound with dissociable substituents highly depends on the pH value too, but this pH dependence fully disappears at higher ion concentrations.

It has been concluded that the structure of the solvate shell around hydrophylic and lipophylic groups is of different ion sensitivity. The monovalent cations

have a higher disturbing effect on solvate shells around hydrophylic groups. The disturbing effect of ions exhibits the order Cs > K > Na for hydrophylic and the order Na > K > Cs for lipophylic groups.

Ethyleneglycol as a Dielectric Model of Hydrated Water

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It has been proved satisfactorily by microwave dielectric investigations that the time of relaxation of water is longer in macromolecular solutions than in pure water.

The frequency dependence of dielectric characteristics of water can be described by a model, according to which water can be separated into two parts: on the one hand, hydrated water of low mobility, the time of relaxation of which is longer than that of free water, and free water on the other.

According to our examinations the own time of relaxation of ethyleneglycol approximates the calculated time of relaxation of hydrated water; therefore, ethyleneglycol solution is suitable for controlling some characteristics of the mathematical model.

Measurement of Vapour Pressure of Bound Water as a Solvent

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It was examined in our previous experiments how the urea and glucose in high quantitites dissolved in the saturated solutions of NaCl and KCl. The saturated NaCl solution was able to dissolve a quantity of urea enough for saturation; a small portion of glucose enough for saturation remained undissolved. Both urea and glucose dissolved in the saturated solution of KCl. Since the hydration envelope of the ions was formed by a portion of water in the solution, part of water can surely be considered as bound water; there is no doubt that the water bound in this way does act as a solvent.

The temperature dependence of vapour pressure of the above solutions was examined in the present experiments. According to our results the boundedness of water increases when urea or glucose is dissolved in saturated electrolyte solutions.

Problems in Standardless X-ray Microanalysis of Biological Thin Samples

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Synaptosomes prepared from rat cerebral cortex were stimulated either with electrical pulses of 2 V, 5 cps, 1 ms, or chemically with 40 mM KCl solution. From these samples smeared preparations were made and studied in a JEOL 100B EM having a JEM ASIC-I scanning attachment. The elementary composition of the individual synaptosomes was determined in spot analysis (80 kV accelerating voltage, 35° tilting angle, 100 – 200 sec analysing time) with a modified EDAX 707B energy dispersive X-ray microanalyser. Mass of the individual synaptosomes was determined by measuring the decrease in intensity of the exciting beam passing through the sample; the intensity of the emitted characteristic radiation was expressed in relation to the mass of synaptosome.

We have examined the effect of some variables (vacuum purity, position of the aperture, beam diameter) on the reproducibility of the quantitative analysis. In our oppinion a successful quantitative analysis of thin biological samples can only be carried out under continuous control of vacuum purity, diameter and intensity of the electron beam as well as the position and tilting angle of the specimen.

Modelling of Gating Currents

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Nonlinear components of transmembrane currents are known as gating currents. Voltage clamped pulses drive a current across the membrane: this current is an exponential function of the time:

$$i = \frac{U}{R} \exp\left(-\frac{t}{RC}\right)$$

where R is the ohmic resistance and C is the capacitance of the membrane. The algebraic sum of the currents brought about by two oppositely directed pulses of equal amplitude is zero if there is no nonlinearity. However, this sum is never zero in case of biological membranes, and the transient difference is thought to be caused by movements of gating particles in the sodium channels. However, one cannot leave out of consideration that the membrane capacitance and resistance can be changed by the displacement of membrane potential, i.e. the time constant

RC is different with oppositely directed pulses. The corresponding currents differ from one another, as proved by calculations and experiments performed with a physical model consisting of a "varicap" diode and ohmic resistors. The difference of the current pulses has a time course similar to that of gating currents. In our opinion this phenomenon should be considered as a possible component of the gating currents.

Modelling of the Summation of Unit Potentials

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In the surface electromyographic recording the potentials represent a compound electrical response of several groups of muscle fibres activated synchronously or asynchronously. The analysis of the EMG records aims at determining the information carried by different motor units. In the present study, a single sciatic-gastrocnemius preparation isolated from frog has been used as a model of one single motor unit. Two or three of these preparations kept in Ringer solution were stimulated with synchronous or asynchronous electrical impulses applied to their nerves and the action potentials of the muscles were recorded.

The results obtained by the analysis of these records are in accordance with previous data concerning the linear summation of the unit potentials.

Landau Phenomenological Theory of Phospholipid Bilayers (Effect of Isotropic Pressure)

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Applying the Landau theory of phase transitions the authors propose a phenomenological model for describing the phase transition of phospholipid bilayers induced not only by temperature but by hydrostatic pressure, too. By means of this model several thermodynamic parameters of different lecithin membranes are calculated (e.g. Clapayron slope, phase transition pressure, -temperature, -enthalpy, -entropy, changes of volume, -membrane thickness, -membrane area per molecule, vertical Young's modulus, gauche number per chain). The calculated values are very well comparable with the measured data.

According to the calculations an increasing pressure causes the phase diagrams of the two-component phospholipid membranes to be shifted linearly to higher temperatures without any drastical deformation in the shape of the phase diagrams.

Charge Mobility in Lipids

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Physical properties of many lipids which compose the biological membranes undergo severe changes upon addition of water. Beside structural alterations the enormous increase in electrical conduction has to be mentioned. This is especially pronounced in the case of small amounts of adsorbed water; under such conditions it covers several orders of magnitude.

Measuring the conduction in alternating electric field of a wide frequency range one can separate the processes going on inside the samples and those at the electrode surfaces. The electrode processes are characteristic of ionic conduction which, in case of lipids, is likely to be a protonic one.

The measurements were made at different humidities of the samples. In all cases characteristic features of electrode events were observed, a finding pointing to the possibility of protonic conduction. From the results of the alternating current measurements it was estimated the mobility of the charge carriers and efforts were made to measure the mobility in a more direct way.

Thermo-osmosis Across the Skin of Frogs

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The revived debate about the nature of osmosis and the latest suggestion of the biological significance of the so-called thermodialysis lay again emphasis on our long-time systemic investigations aiming at clarifying the role of temperature gradient (thermo-osmosis).

The L and Q^* transport-coefficients characteristic of the volume flux (J_v) as well as the J^* "active transport" originated by the skin in Ringer's solution were determined by measuring the change of weight of a sack made of the abdominal skin of *Rana esculenta*. The phenomenological equation approximately describing the phenomenon reads as follows:

$$J_v = L - \Delta\pi + \frac{Q^* T}{vT} + J^* ,$$

where v is the molecular volume of water and $\Delta X = -X_{\text{mucosa}} - X_{\text{serosa}}$. According to our measurements $L = 1.2\ \text{mg} \cdot \text{cm}^{-2} \cdot \text{hour}^{-1} \cdot \text{bar}^{-1}$; $J^* = 1.0\ \text{mg} \cdot \text{cm}^{-2} \cdot$. hour^{-1}. The value of the heat of transfer quantitatively characterizing thermo-osmosis is: $Q^* = 0.8\ \text{l} \cdot \text{bar} \cdot \text{mol}^{-1}$, in contrast to the value of $410\ \text{l} \cdot \text{bar} \cdot \text{mol}^{-1}$, which could be expected on the basis of the vapour pressure conception of osmosis.

Membrane Damaging Effect of Nonionic Surfactants

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Nonionic surfactants (nonyl-phenyl-ethylene-oxide and tributyl-phenyl-ethylene-oxide polymers) considerably influence the effectiveness of other bioactive compounds and also display marked biological activity on their own. For a better understanding of their mechanism of action the lipophility, the adsorptivity, the effects on the ^{42}K-efflux of DPPC (dipalmitoyl phosphatidyl choline) liposomes and on the phase transition of model membranes prepared from DPPC were determined for 19 nonionic surfactants in ion-free and in different ionic environments. It has been found that surfactants increase permeability and, correspondingly, alter the transition temperature and enthalpy. Comparison of the data revealed a good correlation to exist between the biological activity and the above-mentioned changes. In addition, it has been established that the effectiveness of the surfactants depends on the number of the ethylene-oxide groups. We assume that the hydrophilic ethylene-oxide chain interacts with the polar heads of the membrane phospholipids controlling the penetration of the lipophilic alkyl chains. It has been found that monovalent ions decrease the effect of the surfactants on permeability and phase transition.

ESR Study of Primycin Effect on the Structure of Erythrocyte Membrane

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The effect of primycin on the structural order of human erythrocyte membrane was investigated by intercalated fatty acid spin labels.

Concentration dependence was measured in a range of 10^{-6} M -5×10^{-5} M primycin at 20 °C. Primycin drastically decreased the order parameter in this range, while left it nearly unchanged above these concentrations.

Temperature dependence: Primycin (10^{-5} M) decreased the order parameter and the rotational correlation time of spin label between 10 °C $-$ 45 °C. The phase transition temperature of the lipids diminished by about 2 °C.

Time dependence: The decreases of the order parameter and the correlation time appeared as soon as 3 minutes after adding the antibiotic to the erythrocyte suspension and reached constant values in half an hour.

From these results we have concluded that primycin penetrates deep into the hydrophobic region of the membrane and decreases the order of the hydrocarbon chains of surrounding lipid molecules. (The time dependence shows a slight primycin – spin label interaction.)

Influence of Slight Changes in Ionic Content on the Effectiveness of Procaine

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It is an important problem that the ability of local anesthetics to block nerve conduction depends on actual conditions. According to the data in the literature the minimum concentration required to block action potential in frog nerves increases with decreasing pH. The minimum blocking concentration of procaine was measured from pH 6 to pH 8; the effect of pH on Na^+ currents in procaine-treated nerves was investigated under voltage clamp conditions over a wider range of pH.

In our experiments we used sodium hydrocarbonate solution to buffer the Ringer solution to pH 8.3 and determined the minimum blocking concentration of procaine at this pH. However, the minimum blocking concentration depended not only on the pH but the sodium ion concentration, too. When the sodium ion concentration was decreased from 125 mM/l to 115 mM/l in the Ringer's solution the minimum blocking concentration of procaine increased from 0.82 mM/l to 2.8 mM/l. It is our intention to clarify the role of pH and sodium ions in the blocking action of procaine by further experiments with spin-labelled procaine.

Entrapment of Heparin into Liposomes

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It has been the aim of the present work to entrap heparin into liposomes in order to make it suitable for oral treatment. A lecithin–cholesterol mixture was used in the experiments. The entrapment of heparin into liposomes had some difficulties. The heparin binds the positive groups of lipids and prevents the formation of liposomes. In order to avoid this undesirable phenomenon we first tried to mix our lipids with phosphatydyl-serine (a lipid with highly negative head groups), but this procedure resulted in an unstable suspension, in which the vesicles tended to fuse to each other. Probably, heparin caused a lateral phase separation and interacted with the positive surface regions.

In the second phase of our study we changed the ionic milieu of the surrounding medium and obtained in this way a stable liposome suspension.

The third phase of our experiments have brought some indirect evidences that the liposome-entrapped heparin could cross the intestinal barrier of rats in the case of oral administration.

Comparative Biochemical and Biophysical Studies on Rat Brain Synaptosomes

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Synaptosomal preparations obtained by three different methods were compared as to their biochemical and biophysical characteristics.

The fractions were similar to each other in their contamination by other subcellular particles. Physiological activities of synaptosomes were checked by changes in their membrane potential. The membrane potential was monitored by using a potential-sensitive fluorescence dye [3,3′-dipentyl 2,2′-oxacarboxyanine; $diOC_5(3)$] according to the optical method described by Blaustein and Goldring (1975).

The changes in fluorescence intensity as a function of K^+ concentration can be described by the Goldman–Hodgkin–Katz equation. Under iso-osmotic conditions all of the synaptosomal preparations could be depolarized by addition of extra amount of K^+. The rate of depolarization varies but slightly with the type of synaptosomal fractions. Using the same circumstances no changes in membrane potential could be detected in reference fractions.

It is concluded that synaptosomes obtained by various preparation techniques do not significantly differ from each other in their physiological activities.

Interaction of Cytoplasmic Glycerophosphate Dehydrogenase with Model and Mitochondrial Membrane

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Cytoplasmic glycerophosphate dehydrogenase ($GlDH_{cyt}$) has a multiple role in cell metabolism. One of its essential functions is that, as a member of the glycerophosphate shuttle, it provides cytosolic reducing equivalents for the respiratory chain of the mitochondria. As a working hypothesis we assume that $GlDH_{cyt}$ can bind to the mitochondrial membrane. This assumption is supported by the following experimental data:

(1) $GlDH_{cyt}$ enzyme activity is enhanced in the presence of either egg yolk lecithin liposomes or mitochondrial suspension. (2) The binding of NADH to the enzyme is also influenced by the presence of liposomes. (3) The molecular ordering

of the egg yolk lecithin liposomes, as measured with intercalated spin probes, is altered, due to $GlDH_{cyt}$–membrane interaction.

The above findings are interpreted by assuming that $GlDH_{cyt}$ can bind to the membrane surface as a peripheral protein and this binding alters its catalytic properties making thereby the shuttle more effective.

The Effect of X-radiation on the Surface Morphology of Human Embryo Fibroblasts

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The dose- and time-dependent morphological effects of X-radiation on human embryo fibroblasts have been examined by scanning electron microscopy. The control cells in confluent culture had smooth surface and were well flattened. Ten minutes after irradiation the cells showed an extensive ruffling activity and extension of filopodia. The cells became longer and thinner as compared to control. The contact of cell-to-cell, as well as cell-to-substrate decreased. Later on, (1 – 4 hours after irradiation) this ruffling activity gradually decreased. By the 24th hour the cells were again greatly flattened onto the substrate and had restored smooth surface. Cell-to-cell contact returned to control state. The changes in shape and surface morphology of the cells were induced with a dose as low as 25 rad.

Comparative Investigation of the Effect of Endotoxin and Radio-detoxified Endotoxin on Cell Membranes *in vitro*

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The effect of endotoxin prepared from *E. coli* as well as that of radio-detoxified (150 kGy) endotoxin on membranes of human blood cells were investigated *in vitro* under normal conditions and during X-irradiation with 1 and 5 Gy. The changes on cell surfaces were followed by ^{3}H-concanavalin A lectin-binding technique (G. J. Köteles et al., Nature, *259:* 507, 1976; T. Kubasova et al., Proc. IV. Inst. Congr. IRPA, *4:* 1203 – 1205, 1977; Int. J. Radiat. Biol., in press, 1981). None of the preparations influenced the lectin-binding capacity of erythrocytes in concentrations from 1 to 50 μg per ml. A well-detectable difference, was however, found between the effects of two the substances in the case of platelets and lymphocytes. This held true also when the cells had been treated with the agents before the irradiation. The results suggest that the radio-detoxified

endotoxin prevents the developing of radiation-induced functional perturbation of the plasma membrane to a greater extent than does parent endotoxin. The alterations of the cell surfaces could also be detected by scanning electron microscopy.

Functional Receptor Alterations in Radiation-perturbed Plasma Membranes

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The radiation-induced early and temporary structural and functional rearrangement of plasma membranes alters several membrane-bound features (cell motility, surface charge, enzyme activities and quantity of surface antigens, etc.; G. J. Köteles, At. En. Rev., *17:* 3 – 30, 1979). Previously, we have demonstrated dose-dependent changes in ^{3}H-concanavalin A binding sites (non-specific receptors) on the surfaces of human fibroblasts, platelets, lymphocytes and erythrocytes in the dose-range of 0.1 – 9 Gy (G. J. Köteles et al., Nature, *259:* 507, 1976; T. Kubasova et al., Proc. IV. Int. Congr. IRPA, *4:* 1203 – 1205, 1977; Int. J. Radiat. Biol., in press, 1981). In the present paper we bring evidences for temporary alterations also of specific virus receptors, i.e. attachment of polio live vaccine (type 2) to primary cultures of green monkey kidney cells upon the acute effects of two different radiations, tritiated water (37 kBq per ml) and X-irradiation (2.5 Gy). It is suggested that the temporary alterations of plasma membranes might contribute to the development of early and late effects of ionizing radiations.

Investigation of F_c Receptors of Human Monocytes with the Use of Flow Cytometer

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In human peripheral monocytes two subpopulations can be distinguished according to their size determined by scattered light intensities of the cells. The number and the binding constant of F_C receptors of small and large monocytes were determined with flow cytometer (FACS III) using FITC-labelled monoclonal IgG_1. The binding constants in the two subpopulations were identical within the experimental errors [$K = 1.2 \pm 0.3 \times 10^5\ M^{-1}$]. The number of receptors was significantly different; the small monocytes possessed $3.3 \pm 0.6 \times 10^5$ while large ones $10 \pm 1 \times 10^5$ F_C receptors. When the different sizes of the cells were also taken into account the receptor density of large monocytes proved to be twice as

high as that of the small ones. The paper discusses the advantages (reduced time demand, increased reliability, wider applicability and greater information content) of receptor investigations performed with flow cytometer in detail, in comparison with receptor determination by means of methods using radioactive isotopes.

Preparation of Microvessels from the Spinal Cord: Characterization of the Subcellular Fraction

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In this paper attempts have been made at isolating microvessels of high purity from the spinal cord. The procedure used followed the main principles previously described for the isolation of capillaries from cerebral cortex but, due to the special structural characteristics of the spinal cord, the mechanical dissociation was carried out by pushing the chopped tissue through metal sieves 1.0 and 0.75 mm in pore size. This was followed by low speed and sucrose density gradient centrifugations. Microvessel fraction was studied by light-, scanning- and transmission electron microscopy. Alkaline phosphatase and γ-glutamyl-transpeptidase activities – marker enzymes for capillaries – were determined in biochemical studies.

The availability of a subcellular fraction enriched in microvessels renders possible to study with biophysical and molecular biological methods those transport processes, which are involved in the supply of nutritive substances to the spinal cord.

Kinetic Changes in the Lipid Components of the Liver Cells of Mice under the Effects of Selenium-Methionine Dosage

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As known, lipids together with the albumins represent the main components of the cell membranes. This means that the changes in these components and in their properties may exert profound influence on the structure and functions and on the regulatory functions of the membranes in particular.

In the case of a rather great variety of radioprotector oxydants the radiation protecting effect is generally represented by the normalization of the level of the antioxidizing activity of the lipid components or, by normalization of such biochemical processes which are not otherwise characteristic of the same organism.

Starting with this fact we have investigated the effect of administration of selenium-methionine on the lipid components of liver cells.

We have studied the composition of the lipid fractions of the liver cells in white mice (Balb) by means of thin-layer chromatography 3, 18 and 24 hours after the administration of selenium-methionine.

The neutral and polar components of the lipid fractions have been determined. Selenium-methionine treatment causes quantitative as well as qualitative changes in the lipid fractions. The temporal changes in the components of the lipid fractions coincide with the changes in the antiradical activity of the fractions.

Regulation of K^+ Transport in Wheat Seedlings

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The changes in the regulatory mechanism of the K^+ transport process was investigated in wheat seedlings from the time of germination till the two-leaves stage of development. During this period (10days) the K^+ influx, long distance transport and K^+ content of the plants were followed. The onset of changes in the type of nutrition from embryonic to adult form was found to depend on the K^+ concentration of the complete growth medium.

In excized roots the K^+ influx rate was determined by the K^+ content of the roots. Low K^+ content was coupled to high influx rate and *vice versa.*

The extent of long distance transport from root to shoot was a function of the K^+ content and showed two maxima. The characteristic features of the two peaks were analyzed by using inhibitors of respiration and photosynthesis. It seems that one of the maxima is coupled to photosynthesis, while the other one to respiration.

This work was supported by the Department of Plant-protection and Agrochemistry of the Ministry for Agriculture and Food (MÉM NAF).

Induction of Nitrate Reductase in Wheat

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In higher plant nitrate reductase (NRase) is a substrate inducible enzyme. The induction can be blocked by inhibitors of protein and nucleic acid synthesis a finding suggesting a *de novo* synthesis of NRase.

In order to determine the most effective NO_3^- concentration in NRase induction NRase activity was followed in wheat seedlings growing in complete growth medium of various NO_3^- concentrations. Induction of NRase activity was found to require a NO_3^- concentration of at least 5×10^{-4} M. It reached maximum value at a concentration of 3×10^{-3} M.

The influence of the end product, i.e. ammonium, on the NRase activity was studied in plants grown in a series of growth media containing NO_3^- and NH_4^+ in different ratios.

During ontogenesis NRase activity is influenced by both the NO_3^- level and the age of the leaf and plant. A vertical gradient of NRase activity along leaf levels was observed: the flag leaf possessed high NRase activity during ear formation as compared to the lower leaves.

This work was supported by the Department of Plant-protection and Agrochemistry of the Ministry for Agriculture and Food (MÉM NAF).

The Effects of pH and Ca^{2+} on the K^+ Influx of Rice and Wheat Seedlings

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The influence of pH and Ca^{2+} on K^+ influx of rice and wheat seedlings grown under controlled conditions was studied at room temperature. Using fatty acid spin labels, the ordering state of the membrane lipids in the plasmalemma-rich microsomal fractions (PRMF) prepared from rice and wheat roots was also measured. Membranes were kept in a Tris-MES buffer solution (pH = 3 – 8) between 0 and 30 °C in the presence and absence of Ca^{2+}. The ordering state of lipids was characterized by 2A,, values calculated from ESR spectra. It was observed that

- decreasing the pH from 7 to 3 increased the K^+ influx and decreased the K^+ content of rice roots; in the case of wheat roots, the K^+ influx and K^+ content were independent of the pH;
- the K^+ influx of rice roots became independent of pH at a certain Ca^{2+} concentration;
- elevating the concentrations of H^+ and/or Ca^{2+} increased the molecular ordering of lipids in the rice PRMF;
- in the case of the wheat PRMF, the 2A,, value was higher in the presence of Ca^{2+} ($c = 3 \times 10^{-3}$ M) at all pH values and the ordering state of lipids was found to be independent of the pH.

The results are explained in terms of active and passive K^+ transport processes.

Inhibition of K^+ Absorption by Heavy Metal Ions

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It has been the aim of the present paper to investigate the inhibition by heavy metal ions (e.g. Cu^{2+}, Cd^{2+}, Hg^{2+}) of the metabolism-dependent K^+ absorption system of excized wheat roots. Concentration dependence, time course and type of the inhibition as well as the recovery processes have been examined. The inhibition can be diminished when chelating agents are present together with the heavy metal ions.

The inhibition caused by a short-time (30 min) Cu treatment increases during subsequent washing in a Cu-free solution, even in solutions containing a chelating agent (Na_2EDTA). In the presence of cysteine there is no increase of inhibition during the washing period. After one hour, the inhibition of K^+ absorption can be diminished also by washing in control solutions. The effect of Cu was compared with that of other heavy metal ions. During the metal-free washing period a similar increase in the inhibition could be observed with Cd^{2+}, too. The inhibition due to Cr^{3+}-, Fe^{2+}- or Hg^{2+} treatment either remained unaltered or decreased. Washing the excized root with cysteine-containing solutions restored K^+ absorption to a greater extent after Hg^{2+} than after Cu^{2+} treatment.

Effect of Metal Ions on Active Calcium Transport in Human Red Cells

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The active calcium transport of the plasma membrane plays a fundamental physiological role in most living cells. Preparation of inside-out human red cell membrane vesicles (IOVs) allows one to study the substrate specificity and regulation of the ATP-dependent active calcium transport *in situ*.

In this report we demonstrate that certain metal-ATP complexes (such as MgATP, MnATP, CoATP, NiATP and FeATP) are able to serve as substrates for the calcium pump in IOVs, while others (such as SrATP, BaATP, CuATP, CdATP and trivalent cation-ATP complexes) are not. Calmodulin activation of calcium transport does not depend on metal ions used for the activation of the pump. Mn, Co and Fe significantly enhance calcium-dependent, hydroxyl-amine-sensitive phosphorylated intermediate (EP) formation in the presence of

γ-^{32}P-ATP. Mg and Ni have no, or just slight, effect on this reaction. Our experiments indicate that Mn, Co, Fe and Ni can substitute for Mg in the dephosphorylation of the calcium pump enzyme.

Effect of Beta Radiation on Na^+ and K^+ Transport of Muscle

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The authors' previous investigations on the effect of different dosages of beta radiation on Na^+ and K^+ transport as well as on Na^+ and K^+ content of heart muscles and striated muscles have been continued. Isolated frog hearts and striated muscles (M. sartorius) were irradiated with ^{90}Sr beta radiation source in normal Ringer's solution containing $^{42}K^+$ and $^{24}Na^+$. Non-irradiated preparations treated in the same way served as control. Effects of six different dosages between 10 to 120 Gy were examined. The results were evaluated on the basis of data of 170 experiments. The Na^+ and K^+ content does not significantly change upon the effect of the dosages applied. Within the dose range from 10 to 30 Gy the Na^+ transport did not differ from that of the control preparations, while upon the effect of 40 and 60 Gy the Na^+ transport of heart muscles increased significantly. These data also indicate that the radiation-sensitivity of heart muscles is higher than that of the striated muscles, and that the effect reaches the extracellular field of the tissues examined.

Investigation of the Intraocular Tension and Aqueous Humor Production by ^{131}J Albumin in Rabbits (The effect of Timolol eyedrop)

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The author has studied the local effect of Timolol on the rabbit eye. The intraocular tension and the aqueous humor production were measured by ^{131}J albumin.

The decrease of the intraocular tension after Timolol treatment might be assumed to be due to a decrease in the aqueous humor production.

Comparison by UV Difference-spectroscopy of the UV-Damage to T7 and MS2 Phages

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In our previous investigations a UV difference spectroscopic method was used to follow the effect of UV light (254 nm) on T7 phages and isolated T7 DNA, respectively. In the present work the investigations have been extended also to MS2 phages and MS2-RNA.

A distinct diminution of absorption was found near 260 nm in the spectra of irradiated isolated MS2-RNA and MS2 phage, with a similar dose-dependence in both cases; this was explained by the production of pyrimidine-photohydrates and -dimers. Besides, at higher UV doses (> 5 kJ/m^2), a sharp decrease was found in the absorption of MS2 phages at 225 nm which could be explained by formation of an E-N(2-oxo-pyrimidyl-4)-lysine-type cross-link.

Comparing the dose-response curves of isolated T7-DNA and MS2-RNA we have established: (1) an irreversible production of uracyl-hydrates in MS2-RNA; (2) at higher UV doses (> 5 kJ/m^2) the main photoproduct may be the protein – nucleic acid cross-link in both phages; (3) the type of cross-link can be judged from the UV difference spectra.

Effect of Laser and Conventional Light on Bacteriophages

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It has been demonstrated in our previous investigations that laser light of extremely high power may exert biological effects which could not be observed till now with the use of conventional light sources. It is the aim of the present investigations to compare the biological effect of laser light and of conventional light of low average power, the incident light of which is absorbed in phage-nucleoproteid systems.

The biological objects used were T7-bacteriophage and MS2 phage photosensitized by 8-MOP. In case of the T7 phage we used a nitrogen laser pumped by KDP crystal frequency-doubled coumarin dye laser as laser light source while the conventional light source was a Xe lamp (280 nm). In case of the MS2 phage sensitized by 8-MOP the nitrogen laser and filtered mercury vapour lamp (337 and 334 nm) were used as light sources.

We determined the dose-response curves as well as the inactivation cross sections from the initial slopes of the curves. We established and confirmed quantitatively that the biological inactivating effect of the laser light and that of conventional light were identical within the measurement errors.

A New Device to Measure UV, VIS and IR Radiation: Some of its Application Fields

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In the quantitative photobiophysical and photochemical experiments carried out in this institute it is necessary to know exactly the energy flow rate (power density in W/m^2) of the various light sources. For this purpose we have developed a new radiation measuring instrument.

Advantages of our equipment:

It has several measuring heads which enable the measurements to be performed independently of the wave length and make it appropriate for internal calibration.

By the use of the equipment we have obtained experiences in the following areas:

1. During comparison of the effect of laser and conventional light the light parameters were measured by this equipment.
2. It was used for measuring the spectra of the Yvon Jobin's spectrofluorimeter. By a special measuring head we could directly measure the whole quantity of incident light.
3. We were able to measure the parameters of a high-power Xe light source of type Yvon Jobin and also those of a monochromator.

Application of VUV Spectroscopy for the Study of Intact Bacteriophages

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A new method has previously been published for preparation of native, high quality DNA thin films for optical measurements which extends DNA spectroscopy down to 164 nm. In its original form this method is suitable for preparation of phage films with partially denatured protein coat; in its developed form it can also be used for preparation of intact phage films. In the present work the latter types of T7 phage films were investigated in the VUV region and compared with isolated T7-DNA. The local denaturation of the protein coat could be detected by the VUV spectra. At the same time, the DNA remained in native form (B conformation) during denaturation of protein as shown by the hyperchromicity (20 – 30 per cent) obtained at 260 nm after heat denaturation.

The difference in UV damages (254 nm) to whole T7 phage and isolated T7 DNA found earlier was also detected in the VUV spectra. Due to the DNA – protein cross-links, the increase in absorption at 185 nm which is characteristic of pyrimidine dimers could not be detected in the case of whole T7 phages. Instead of this, a decrease of absorption was observed in the whole range from 300 to 160 nm; this decrease corresponds to the cross-link formation.

A Reaction Kinetic Model of UV damage to T7 Phages

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A reaction kinetic model was developed to explain the UV damage to phages T7 quantitatively.

Logically, three types of damageable sites were assumed in the model:

(i) single bases which can form cross-links,

(ii) base pairs which are capable of forming dimers of adducts only,

(iii) base pairs which can also form DNA protein cross-links.

The comparison of the model and the experimental results shows that, in the case of T7 phages, there are only two types of damageable sites: *(i)* and *(iii)*.

The only fitted parameter of the model is proportional to the rate constant of cross-link reaction. Two other parameters were also used which were obtained from another model of the UV damage to isolated T7 DNA.

Circular Dichroism to Study the "*in situ*" Conformation of Nucleic Acids

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The conformation and, consequently, the reactivity of nucleic acids in natural milieu can differ from the related properties of isolated nucleic acids in several aspects.

Optical activity as a sensitive characteristic of the conformation of nucleic acids has been investigated in relatively simple nucleoproteid systems (bacteriophages). Circular dichroism of bacteriophages containing single-stranded DNA (OX174), double-stranded DNA (T7) and single-stranded but in 80 per cent base-paired RNA (MS2) has been measured. Spectra of isolated nucleic acids have been compared with the well separable parts of the whole phages spectra specific to nucleic acids.

Circular dichroism has been found to be a sensitive method for the detection of slight conformational changes produced by environmental factors.

Microwave Radiation: Biological Effects and Dosimetry I. Dosage of Microwaves

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The experimental study of biological effects of microwaves has been started in our institute in 1979. It has seemed of outstanding importance that experimental results be reproducible and adequate for comparison with standard international data. Based on data in the literature, however, exact conditions of microwave dosimetry cannot be considered as defined at all. Recent works indicate parameters which, unusually for those working with ionizing radiations may affect the entering of microwave energies into organs and their absorption in tissues. In this respect, we deal with: the interaction of microwaves with the material; the decrease of transmitting radiation; penetration degree; absorption cross section; distribution of the dissipated energy in the organ; dosimetry measurements (power density, absorbed dose). Experiments were performed under cavity resonator and free space irradiation conditions, respectively. In addition to measuring power density, the specific absorbed rate was determined with the aid of a thermistor measuring system. The rate of maximum and minimum heating capacity occurring in the area of irradiation was established just as was the total capacity value of microwaves dissipated in the embryonal eggs examined.

Microwave Radiation: Biological Effects and Dosimetry II. Biological Effects on Chick Embryos

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Since the radiosensitivity of the embryonal organism is higher than that of the adult one, white Leghorn chick embryos were used in the examinations (incubation: 37.5 ± 0.5 °C, 70 per cent humidity). Continuous microwave irradiation was applied at 2450 ± 50 MHz frequency (CW) in 100 mW-2 W/cm^2 power density range. The embryos examined were irradiated with increasing power density. Survival, rate of development and formation of differentiation were investigated at various points of time of the embryogenesis (from the 2nd to 19th the day). The various stages of the embryonal development were found to show unlike radiosensitivity. Aiming at comparative examination of the protein synthesis of embryos irradiated on the 15th day of embryonal development (70 per cent survival), tRNAs and aminoacyl-tRNA synthetases were isolated from the brain

and liver. Measurements performed on tRNA acceptor activity and on enzyme activity of the synthetases showed some decrease in the liver when compared with the control, while an increase was observed in the brain in 70 – 80 per cent of the cases. The elucidation of the mode of action needs further examinations.

Use of the Bioenergetic Effect of LASER Irradiation for Treatment of Duodenal Ulcer

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According to earlier observation on the LASER's effect, polarized radiation induces a non-thermal (bio-stimulatory) influence that promotes wound healing. As the treatment is still an unsolved problem we have attempted to use the Ag^+ ion LASER irradiation for therapy of the duodenal ulcer.

The LASER beam went through a quartz fibre planted into a teflon catheter that led through the manipulation channel of an Olympus TGF operation endoscope. It was an aimed, eye-controlled treatment with the use of a small-energy LASER beam (5 – 10 Joule) which was applied at 0.5 – 2 W continuously or interrupted in each 0.5 sec.

Ulcers causing severe complaints for several years and resistent to other therapy, as well as slowly recovering ulcers were selected for the treatment. The treatment was weakly repeated under endoscopical control once or several times until full recovery. During LASER therapy the patients were off-diet and received sodium bicarbonate but occasionally, without any other drug treatment. The endoscopical observation showed a faster recovery, and the complaints disappeared during the first three days of the treatment. It is suggested that the non-thermal, bio-stimulatory effect of the LASER gives a new opportunity for the therapy of duodenal ulcer.

Fission Neutron Irradiation of Mice Embryos in utero I. Physiological and Biochemical Changes Measured *in vivo*

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Female $C_{57}Bl$ mice were exposed to single whole-body dose of 0.5 Gy fission neutrons on the 17 – 19th day of pregnancy. There was a slight decrease in body weight of newborn animals, and 189 out of 445 died during the first three

days after birth. The weights of liver, kidney and testis were slightly decreased till to the 36th day. Brain weight was 80 per cent of the control on the 20th day.

Intensity of DNA, RNA and protein synthesis was measured *in vivo* by incorporation of ^{14}C-labelled precurcors in three-week-old mice, i. e. at the time point of the marked change in brain weight. There were no change in the incorporation into DNA and RNA while the amino acid incorporation into the acid soluble proteins decreased by 25 per cent in the liver and by 40 per cent in the brain. Acid soluble proteins were separated by acid urea polyacrylamide gel electrophoresis. Synthesis of histones decreased markedly and phosphorylation of histones was 50 per cent lower in the irradiated than in the control group.

The experiments suggest that the marked physiological changes in the brain upon the effect of fission neutron radiation can primarily be attributed to a damaged synthesis of proteins and not that of nucleic acids.

Effect of Fission Neutron Irradiation on Mouse Embryo II. Changes of Cell-free Protein Synthesizing System Isolated from Brain and Liver

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Mice previously exposed to an 0.5 Gy fission neutron whole body irradiation intra-uterinally on the 17 to 19th days of pregnancy were killed at the age of three weeks, and the protein synthesis of the brain and liver was examined in isolated cell-free systems (mitochondria, microsomes, pH5 fraction, ^{14}C-amino acids, ATP, GTP, creatin-P + kinase). Incorporation rate of labelled amino acids did not change in the liver mitochondria, while the brain mitochondria of irradiated mice showed about 20 per cent higher biosynthetic activity than did non-irradiated controls. In the cytoplasmic (non-mitochondrial) system the activity of microsomes and that of the adaptor system (tRNAs + ligase = "pH5-fraction") were examined separately by measuring amino acid incorporation and tRNA-amino acetylation, respectively. In the presence of non-irradiated pH5 fraction brain and liver microsomes isolated from irradiated mice did not show any significant changes.

Nevertheless, tRNA-amino acetylation ability of the pH5 fraction isolated from the brain and liver showed of significant decrease upon the effect of irradiation. The decrease in the function of the adaptor system affects also the activity of microsomes as proved by compilation of systems of various combinations. Based on the results presented it is probable that the disturbance of brain protein metabolism of intrauterinally irradiated mice can be attributed to an injury of the soluble components of protein synthesis.

Gene and Chromosomal Mutations Induced by Tritium in Cells Cultured under Chronic Exposure Conditions

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The amount of tritium present in the environment, its expected increase, as well as the biological behaviour of the radionuclide all indicate the importance of a better understanding of the radiotoxicity related to it. In model experiments the gene- and chromosomal mutations induced by tritium used in two chemical forms (tritiated water, HTO; and tritiated thymidine, ^{3}HTdR) were studied on Chinese hamster ovary (CHO) cells cultured *in vitro* under chronic exposure conditions. Cells were kept in culture media containing HTO or ^{3}HTdR with a radioactive concentration of 3.7 kBq – 37 Bq per ml for over a year. During the incubation period, the gene mutations induced on hypoxanthine-guanine-phosphorybosyl-transferase ($HGPRT^-$) in the 6TG selective system and proline loci, furthermore, the structural and numerical chromosome aberrations were determined. According to the results obtained higher radioactive concentrations of tritium (3.7 kBq/ml) HTO and 370 Bq/ml ^{3}HTdR, respectively) cause increased occurrence frequencies in all mutations tested, while the lower concentrations did not produce any alteration as compared to the control, unexposed cells.

Effect of Acute X-ray and ^{60}Co-Gamma Irradiation on Prostacyclin Producing Ability of Rat Arteries

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Clarification of the radiobiological role of prostaglandins is of importance since the biological effects of some prostaglandins exhibit many features similar to, and identical with, the phenomena observed in some phases of radiation sickness. The membrane injuries induced by ionizing radiation and the acceleration of lipid-peroxidizing processes significantly influence the prostaglandin synthesis, too. The clarification of changes induced by ionizing radiation in prostacyclin (PGI_2) – thromboxan (TxA_2) system is of high importance in haemostasis; it might solve the problem of the occurrence of post-irradiation thrombo-haemorrhagic complications.

The authors performed thrombocyte aggregation bioassay examinations on the prostacyclin producing ability of male rat arteries after acute, lethal X-ray and ^{60}Co-gamma irradiation.

It has been concluded that

1. Prostacyclin synthesis in the arteries highly depends, even under normal conditions, on the type of the artery, the structure of the arterial wall, the calibre of the artery and the age.

2. Following lethal X-ray and ^{60}Co-gamma irradiation the prostacyclin production underwent various alterations (increase and decrease, respectively) depending on the age, the type of artery and the time elapsed from irradiation.

Optimization of Irradiation of Tumours

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Successful radiation treatment of deeply located tumours with high-energy γ-sources requires exact medical and physical treatment planning. The computers were the first to offer a possibility to make individual irradiation plans for each patient.

The first programs could only determine the dose distribution in the body, produced by given physical parameters of the irradiation. The next aim was to develope optimization methods for the determination of parameters, which produced optimum dose distribution. At the present there are only a few programs, which are able to estimate several parameters simultaneously; and even those require a rather long computing time.

In this lecture we present our optimization method. It is based on the principle of least squares, and can simultaneously determine nearly all of the physical parameters of irradiation. The dose distribution and its derivatives are computed by a semi-analytical model specially elaborated for this purpose.

We present the first results of our program and discuss the first experiences as well as the limitations of the method.

Delayed Radiation Injury of the Brain Caused by Beta Irradiation

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The beta-irradiating ^{90}Yttrium (half-life time: 64.2 hours) is used in stereotaxical neurosurgery to produce circumscribed lesions in the brain.

The hystological reactions caused by beta irradiation (necrosis, brain oedema) are well known, but there are few information concerning the late reactions of the brain tissue.

In our experiments active (0.125 – 1.0 mCi) and inactive ^{90}Y rods were implanted into the deep white matter or epidural space of cats and dogs. The time between implantation and sacrification varied between 4 months and 3 years.

The brains of the animals was examined with light and electron microscope. An active and remarkable break-down process of myelin was found even as late as 3 years after the implantation.

The net result of the above-mentioned process is a circumscript demyelinated area resembling multiple sclerose plaques. The characteristics of primary and secondary demyelinizations were proved electron microscopically.

Examination of ^{144}Ce Retention in Whole-Body Irradiated Mice Treated with Complex-forming and Radioprotective Compounds

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The occurrence of ^{144}Ce isotope in the fission products formed in nuclear reactions is about 4.5 per cent. Since the features of this radionuclide are still inadequately revealed animal experiments were performed on the deposition-modifying effect of ^{60}Co-gamma and neutron + gamma whole-body irradiation, the retention-effecting ability of different decorporating agents (Ca-DTPA; DFOA; salicylic acid, SA), of radioprotective compound (AET) and of their combinations.

The time-change of the activity in experimental animals was followed by means of small animal whole-body counter. Whole-body measurements revealed that the excretion of ^{144}Ce from the organism showed a retention-time correlation defined by the sum of two exponential functions.

DFOA and AET did not influence the retention of ^{144}Ce while, after application of DTPA, DTPA + SA and DTPA + DFOA + SA combinations the amount of excreted isotope was significantly (by 60 – 70 per cent) increased.

It was also established that application of the LD_{50} of ^{60}Co-gamma and neutron + gamma whole-body irradiation raised ^{144}Ce excretion by about 5 – 10 per cent; however, the decorporation rate could be increased by applying complex-forming compounds.

Radioresistance Transfer by R Factor

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R46 R factor was transferred to wild-type *E. coli K12* strain, *E. coli AB 1157* and to its rec mutants: $recA^-$, $recB^-$, and $recA^-$ $recB^-$ double mutants by conjugation. The appearance of R46 R factor in rec^+ and rec^- *E. coli* strains was

detected by conferring resistance to ampicillin, streptomycin, tetracyclin and sulfonamids.

R46 R factor slightly increased the survival of the wild-type strain and had no effect on the survival of recA$^-$ and recA$^-$ recB$^-$ double mutants.

A well-detectable increase in UV and ^{60}Co-gamma resistance was found in recB$^-$ mutant harbouring R46 R factor. It seems that R46 R factor is able to partly compensate the lack of the encoding activity for exonuclease in recB$^-$ mutant.

Reflection in the Peripherial Blood Picture of the Injury and Regeneration of Bone Marrow after Low-dose Irradiation

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Blood cell counts and indices for bone marrow haemopoiesis (CFU-S and CFU-C) were studied parallelly for fourteen weeks after 0.5 Gy acute gamma irradiation. Although the injury of bone marrow could be detected in the peripheral parameters too, no parallelism was found in the damage and in the restitution of the bone marrow and of the peripheral blood. Most characteristic differences were found during the regeneration: peripheral parameters and bone marrow CFU-S and CFU-C levels oscillated in a cyclic fashion but with phase-differences in the cycling pattern.

These results suggest two conclusions:

1. The restitution of the haematological effects of low-dose irradiation is a relatively slow process;
2. After slight bone marrow damage the functional state of blood forming can be correctly evaluated only on the basis of repeated studies of the blood picture.

Investigation on the Radioprotective Effect of Quinoline-framed Compounds

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From some ten years ago on, some American authors (Westland et al.) wrote about quinoline-framed compounds that contained S-2(alkylamino)ethyl- -H-thiosulfat, 3-alkylthiazolidin and 2-pyridyloxy-derivatives and their analogues

as substituent on the second carbon atom. Some of these compounds proved to be efficient radioprotectors.

In Hungary also Bär et al. (1971) reported on quinoline-framed compounds with antioxidant and radioprotective effects. Some of these compounds were radiosensitizers. As regards structural changes it was found that the substitution was located on the sixth carbon atom, in contrast to the above-mentioned American radioprotectors. The experiments aimed at investigating the radioprotective effect of Kontrad (= 6,6′-methylene-bis)-2,2,4-tri-methyl-1,2-dihydroquinoline disulfone acid natrium). The toxicity of the compound is very low ($LD_{50/72}$ = 4000 mg/kg), optimal dose is 1 g/kg, i.p. (!) in mice. Its radioprotective effect is more favourable than that of AET for it can be applied in a subtoxic dose; however, the DRF is lower than that of AET. The results obtained with post-irradiation application of Kontrad are promising.

Effect of Radioprotective Compounds on Brain Serotonin Level

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Ionizing radiation of the organism evokes obvious primary changes in the processes of the central nervous system. These processes can be detected either by different pharmacological methods or by the changes in neurotransmitter levels measured by biochemical methods.

The authors measured with spectrofluorimetric method the level of serotonin 5-HT and of its metabolite, 5-hydroxyindole acetic acid (5-HIAA) which play an essential role in the neurotransmission of the CNS.

It was concluded that cerebral 5-HT level of experimental animals exposed to lethal dose of ionizing radiation highly decreased while the amount of the metabolite increased.

The increased 5-HT and decreased 5-HIAA levels caused by different radioprotective compounds (AET, cystein etc.) containing SH-groups, are due to the reversible inhibition of monoaminooxydase enzyme.

Book Reviews

An Introduction to Physical Properties of Large Molecules in Solution by E. G. Richards. In IUPAB Biophysics Series, Editors: Franklin Hutchison, Watson Fuller and Lorin J. Mullins, 266 pages with 52 figures, and with an additional chapter on "The Scattering of Radiation by Macromolecules" Cambridge University Press, Cambridge—London—New York—New Rochelle—Melbourne—Sydney 1980.

This is an elementary textbook for the non-specialist about the nature and behavior of large molecules in solution. It is based on a course of lectures delivered to third year undergraduates in physics, chemistry and biology. The text assumes only a minimum background knowledge of mathematics, physics and chemistry. The tone is theoretical and little emphasis is laid on experimental techniques are described. The treatment of problems is rather general and is not concerned with individual molecular properties and chemical structure. Much more space is devoted to models and theories than to experimental observations. The 9 chapters of the book are more or less independent units.

Chapter 1 is a brief summary of the nature of macromolecules and colloidal particles, and touches upon the chemical structure, size, shape and molecular weight distribution of polymers.

Chapter 2 is a more detailed survey of molecular interactions and the physical nature of secondary forces. Intramolecular, interactions and the effect of solvent upon them are analyzed. Multipole interactions, induction forces, dispersion forces, exclusion forces, hydrogen bond, hydrophobic bond are all discussed.

Chapter 3 is devoted to the equilibrium thermodynamics of polymer solutions. In this chapter extensive, intensive and partial properties are defined, and the macroscopic and molecular properties of the macromolecular solute are related. It is also shown how information concerning molecular architecture can be derived from experimental measurements conducted at equilibrium.

Elementary configurational statistics of linear polymers is given in chapter 4. Bond lengths, bond angles, dihedral angles and conformational parameters are introduced and some dynamic aspects of polymer conformation are stressed. Random coil, helical and globular conformations are surveyed.

A short chapter, Chapter 5, deals with helix-coil transitions and denaturation. The approach is thermodynamic and a specific paragraph is devoted to denaturation and renaturation of proteins.

Chapter 6 is about gels and polymer networks, and the treatment includes structural and energetic aspects.

Chapter 7 written by S. D. Dover — is a good summary of scattering of radiation by macromolecules. It includes the physical background of the mechanism of scattering and describes the principles behind the experimental measurement of light scattering, low-angle X-ray scattering and neutron scattering.

Chapter 8 is a concise survey of the hydrodynamic properties of macromolecules. Basic theory, physical background of the experimental techniques and a guide for critical interpretation of experimental parameters are given. This chapter includes viscosity, sedimentation and diffusion. Transport and equilibrium methods are both discussed.

Polyelectrolytes are discussed in chapter 9 and the physical background of the interaction and transport of charged molecules is given. Donnan effect, osmotic pressure, membrane potentials, equilibrium dialysis are surveyed and light scattering, viscosity, diffusion and sedimentation of polyions are discussed. A paragraph is devoted to electrophoresis.

At the end of the individual chapters there are problems to be solved. These are concerned with both the derivation of results and their application. Instructors can use this volume in teaching physical biochemistry since it provides a basic introduction to the concepts used in the description of the behavior and interactions of macromolecules. The author produced a basic book which will not readily become outdated.

P. Závodszky

Environmental Carcinogens — Selected Methods of Analysis. Vol. 2: Methods for the Measurement of Vinyl Chloride in Poly(Vinyl Chloride), Air, Water and Foodstuffs, by D. C. M. Squirrell *A* &. Thain (ed. -in-Chief: H. Egan), XII + 142 p. International Agency for Research of Cancer, Lyon, 1978.

This book consists of four main parts, each one including one to several chapters.

Part I is an introduction to the carcinogenicity of vinyl chloride that sets the stage, by reviewing the pertaining literature, for the further parts.

Part II gives a general review of approaches to monitoring and measurements. The very short first chapter defines the monitoring requirements in the polyvinyl chloride (PVC) industry. The second chapter describes in detail the standard techniques of measurement and monitoring of vinyl chloride. As far as measurement is concerned, the principles of methods based on ionization, spectroscopic methods, methods based on chemical properties, on thermal or electric properties and finally methods involving separation are summarized. With regard to monitoring, individual sub-chapters deal with the measurement of instantaneous and short-term average concentration, area monitoring, long-term average measurements with special emphasis on personal exposure, monitoring of vinyl chloride in plant effluents, in polymers and in foods and food simulants. A very useful subchapter on the preparation of standard mixtures for calibration and testing of methods of analysis concludes this part.

Part III about the methods of analysis is the most informative section of this book for those who look for actual recipees. All descriptions of the eight different methods included contain a definition of the scope and field of application, a hint to useful references, statement of the principle, enumeration of the hazards involved, a very detailed list of the reagents and the apparatus to be used, and all information that is needed to carry out correct sampling. The procedure is then described in a very detailed manner, followed by instructions concerning the method of calculation and notes on the procedure and repeatability. At the end of the descriptions of each method we find a schematic representation of the procedure in question and a statement about the origin of the method.

Part IV in which three standards for calibration and testing are described in much detail is an extremely useful addition to Part III compiled with utmost care that reflects the sense of responsibility of the authors in writing a book on methods the correct use of which has an impact on human lives.

F. Solymosy

IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. 19: Some Monomers, Plastics and Synthetic Elastomers, and Acrolein. 513 p. International Agency for Research on Cancer, Lyon, 1979.

This volume of the IARC Monographs series follows the same pattern of treating individual chemicals as all other volumes of this series do, i. e. synonyms and trade names, structural and molecular formulae and molecular weight, chemical and physical properties of the pure substance, production, use, occurrence, analysis, carcinogenicity studies in animals, other relevant biological data (including toxicity, embryo toxicity, teratogenicity, absorption, distribution, excretion, metabolism, mutagenicity and other short term tests), and studies in humans including case reports and epidemiological studies.

The following chemicals are dealt with: Acrylic acid, methyl acrylate, ethyl acrylate and polyacrylic acid, acrylinitrile, acrylic, modacrylic fibres, acrylonitrile-butadiene-styrene and styrene-acrylonitrile copolymers, caprolactam, nylon 6, chloroprene, polychloroprene, ethylene, polyethylene, methyl methacrylate, polymethylmethacrylate, propylene, polypropylene, styrene, polystyrene, styrene-butadiene copolymers, styrene oxide, tetrafluoroethylene, 2,4- and 2,6-toluene diisocyanate, 4,4'-methylenediphenyl diisocyanate, polymethylene polyphenyl isocyanate, flexible, rigid polyurethane foams, vinyl acetate, polyvinyl acetate, polyvinyl alcohol, vinyl bromide, vinyl chloride, polyvinyl chloride, vinyl chloride-vinyl acetate copolymers, vinylidene chloride, vinylidene chloride-vinyl chloride copolymers, N-vinyl-2-pyrrolidone, polyvinyl pyrrolidone and acrolein. The amplest space, of course, is devoted to vinyl chloride and its derivatives, due to their wide industrial use.

The book is extremely useful for all those who work with any of the substances included, be it their actual testing with regard to genotoxicity or just their use in the laboratory for any type of chemical or biochemical study.

F. SOLYMOSY

Plasticity of Muscle — Proceeding of a Symposium held at the University of Konstanz, Germany, September 23—28, 1979, by Dirk Pette (Ed.) Walter de Gruyter Berlin—New York 1980.

For many years the muscle tissue has been treated as a rather uniform and static system suitable mainly for studying energy metabolism and energy transformation. A new period has been opened in muscle research by the realization of the complexity and variability of fiber composition and some dynamic properties. The recognition of neural modulation of muscle properties has also led to the concept of plasticity. This book contains fourty-three papers presented during the symposium "Plasticity of Muscle" held in Konstanz, Germany from September 23 to 28, 1979. The purpose of this symposium was to bring together investigators from various disciplines in science and medicine who deal with the plasticity of muscle and its underlying mechanisms. The main topics of the symposium were the heterogeneity of the muscle as a tissue, the program of its ontogeny and differentiation, and the regulation of its properties by innervation, usage, hormones and artificial modulation of neural input. The volume begins with a preface by D. Pette, followed by an introduction of A. J. Buller.

Section I includes ten papers on the heterogeneity of metabolic and molecular properties of muscle fibers. Some of the papers deal with the enzymological heterogeneity of single fibers from human and rabbit muscles. Others show the metabolic homogeneity of the fibers innervated by an individual motor unit. Application of immunofluorescence techniques provided a useful approach to determine the distribution of myosin isoenzymes in fibers of adult and developing muscles.

Section II comprises nine interesting papers on muscular development and growth. These reports show the sequence of myosin during myogenesis or regeneration of muscle after experimental injury. The authors used biochemical and immunohistochemical techniques. The other contributions are devoted either to transitions of contractile protein isoenzymes (myosin, tropomyosin) or to changes in the sarcoplasmic reticulum and sarcoplasma during development.

Six papers of Section III deal with nerve muscle interactions during development and upon artificial modulation of the neural input (reinnervation, denervation, cross-reinnervation, cordotomy).

In Section IV two papers are presented about the influence of usage.

The six papers in Section V reveal the effects of different artificial stimulations on the metabolic enzyme activities, sarcoplasmic reticulum, consumption of oxigen or free fatty acids, or on cation distribution and membrane potential.

Section VI treats the mechanisms of hypertrophy and atrophy. This chapter contains seven papers discussing the effects of different factors — hormones, hypertrophy, disuse, denervation — on protein turnover or myosin-polymorphism in skeletal and cardiac muscles.

Section VII includes three papers about the effect of thyroid hormones on different types of skeletal muscles.

This nicely set-up book sums up the latest experimental results of studies of muscle plasticity and will be of great value to biochemists and physiologists as well as to those who are interested in muscle research. The presentation of the volume is admirable.

K. PINTÉR

Biochemistry of Nonheme Iron by A. Bezkorovainy (Biochemistry of the Elements, Series editor: E. Frieden) Plenum Press, New York, London, 1980. XVIII + 435 p.

The role of chemical elements — both metals and non-metals — in biological processes aroused the interest of scientists working in very different fields, including biochemistry, biology and inorganic chemistry. This series, Biochemistry of the Elements, is the recognition of this ever-growing interest.

The first volume is devoted to the biochemistry of nonheme iron because of the versatile and complex chemical and biological role of this metal. (The second volume will discuss the biochemistry of heme iron.)

The author of this volume starts with an overview of nonheme iron biochemistry including also some highlights of the history of iron biology, and showing the distribution of iron in various life forms. This is followed by a discussion of ferrokinetics, i. e. the tracing of radioactive iron following its parenteral administration. Here he deals with compartment models and a probability theory approach to ferrokinetics.

The following chapters (3—6) discuss the metabolism of iron. First the absorption of iron is considered: the mechanism of iron absorption, and the corporal, intraluminal and mucosal factors affecting iron absorption and some aspects of the regulation of this process. Then the author proceeds to the chemistry and metabolism of the transferrins, the iron-binding proteins of circulation. This chapter discusses the physical and metal-binding properties of the transferrins their primary structure, metabolism, further the distribution of iron in human serum transferrin *in vivo*. The next chapter is on the chemistry and biology of iron storage. The physicochemical properties of the ferritins (the principal form of iron storage in the mammalian organism) are discussed in detail, and also the metabolism of ferritin, and the uptake and release of iron by ferritin. Some data on hemosiderin are also presented. In the following chapter the author deals with the mechanisms whereby nonheme iron can find its way into the immature red cells and the pertinent intracellular iron metabolic pathways.

Microbial iron uptake and the antimicrobial properties of the transferrins is the title of Chapter 7. Microorganisms synthesize low-molecular weight chelators which coordinate iron in the environment and return it to the microorganism. These chelators, called siderophores are considered in this chapter: their structure, physical, chemical and biological properties, and their metabolism. The antimicrobial properties of the transferrins are also discussed here.

A separate chapter is on the iron-sulfur proteins, which are the components of various electron transport systems in microorganisms, plants, and animals. The author writes about rubredoxins, ferredoxins, iron-sulfur proteins of mammalian electron transport, and about the role of iron-sulfur proteins in nitrogen fixation. Finally, the properties of complex iron-sulfur proteins (those containing other components such as flavins, molybdenum or heme) are summarized.

In the final chapter of this volume miscellaneous aspects of iron metabolism are presented. Those subjects are dealt with here for which a considerable body of knowledge is available and which have not been adequately treated in the previous chapters. Phosphitin, an egg iron-binding phosphoprotein, the oxygenases, hemerythrins, iron bacteria and metalloserotransferrin are considered here.

The author assembled the material on nonheme iron compounds that occur in biological systems and presented it in a concise and understandable language. This volume can be regarded as the most comprehensive treatment on nonheme iron. It can be highly recommended to advanced undergraduate and graduate students in the life sciences and to medical students. The extensive references at the end of each chapter help the reader find even the very details of points of his or her personal interest by turning to the original papers.

E. KŐRÖS

Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses by Roger H. Kennett, Thomas J. McKearn and Kathleen B. Bechtol (eds) 423 pages 20 reviews, Plenum Press, New York, London 1980

Six years after Köhler and Milstein's paper in Nature on a continuous "hybridoma" cell line producing a monoclonal antibody, many laboratories are making or would like to embark upon programs to make monoclonal antibodies of use in their own research. This book attempts to present, in a coherent fashion the current state of development in hybridoma technology and additional information about what the use of monoclonal antibodies can produce.

The volume is divided into five major sections each of which contains a collection of chapters written by different authors. The first section entitled "Production of specific antibodies from continuous cell lines *in vitro*" gives an excellent coverage of the development and application of rodent hybrodomas as well as an evaluation of the potential use of human limphoblastoid cell lines as a source of human monoclonal antibodies.

The second section deals with the use of monoclonal antibodies to help define immunoglobulin diversity and structure. It includes a chapter about the use of monoclonal antibodies in clarifying idiotypic determinants on mouse antibodies specific for (1 3) dextran. Another chapter presents the work defining the B-cell repertoire with hybridomas derived from monoclonal fragment cultures. The use of hybridomas to localize mouse immunoglobulin genes is shown in the following chapter.

The third, fourth and fifth sections which occupy a major portion of the book are a compilation of reports describing the production and use of rodent hybridoma antibodies in a large variation of situations ranging from definition of T-cell subsets to mapping of viral proteins. There are reports about detection and analysis of human gene products, about the use of hybridomas in enzyme genetics, the production of monoclonal antibodies against human tumor-associated antigens and others. Ten chapters deal with monoclonal antibodies as probes into cellular differentiation and immungenetics. The examples include the application of rat–mouse hybridomas to study the major histocompatibility complex, monoclonal antibody therapy of mouse leukemia, and reports on monoclonal antibodies against influenza virus, rabies virus and streptococcal antigens. These three sections show different approaches by separate laboratories to similar problems. However, the reviewer feels that the same amount of information could have been presented in a shorter way.

Finally, the editors have included an appendix of the methods necessary for hybridoma production and antibody analysis. This appendix is extremely useful in the book for the prospective antibody producer.

This volume can be recommended as a useful handbook to research students and to scientists who wish to apply hybridoma technology in their own field. The typographic presentation of the book is excellent and it is a good value for its price.

P. ZÁVODSZKY

Comprehensive Virology by H. Fraenkel-Conrat and R. R. Wagner (eds) Vol. 17. Methods Used in the Study of Viruses. Plenum Press, New York and London. XVI + 463 p. 1981.

It must have been quite a difficult job for the editors to select the topics to be treated in this volume, because practically all methods used in pure chemistry, biochemistry, pure physics, biophysics, or, for that matter, in biology as such, are applicable to viruses and, necessarily, cannot be squeezed in a single volume of this size. Despite this difficulty the editors seem to have been successful in selecting most of the methods that are used to study the structure of virus particles and its relation to function. The reader should be warned, however, not to expect a recipe-book of methods, a sort of laboratory manual. None of the chapters include methodology s. str. It is rather a reference work in which the underlying principles of individual methods are clearly presented, properly evaluated and then expanded in an individual way depending on the concept, taste and inclination of the author of that particular chapter. The reviewer has the feeling that the book could have been made more useful and valuable by including some examples (even

by setting them apart in the form of an appendix) to show how numerical values actually measured by using a particular method (described in detail) can be applied to arrive at conclusions concerning certain structural and functional parameters of viruses.

Chapter 1 by M. A. Lauffer is a very lucid summary of what he calls biophysical methods in virus research. The underlying principles of viscosity, diffusion, centrifugation, sedimentation equilibrium, chromatography, electrophoresis, osmotic pressure, and light scattering are very clearly presented, and understandable even to virologists with no special training in higher mathematics. This chapter seems to be the most theoretical one in this volume and to lend itself most to the inclusion of some numerical examples just mentioned above. This chapter concludes with a sub-chapter on the theory of electron microscopy, which is a link to the next chapter.

Chapter 2 by H. W. Fisher describes the use of electron microscopy in virology. Here it is indeed materials and techniques (support films, shadowing, staining, cytological techniques, negative staining, spreading techniques with and without monofilms, freeze-drying, freeze-etching, measuring and counting virus particles and components) that are dealt with in terms of references arranged in a logical order and some schematic drawings, and properly evaluated with respect to applicability (survey of results: general characterization of virus particles, capsid structure and virus classification, viral nucleic acids, reconstitution, assembly, virus maturation, growth and replication of viruses).

In Chapter 3 (Structural Studies of Viruses with X rays and Neutrons) B. Jacrot takes a different approach. After a concise description of the physical basis of X-ray and neutron scattering he summarizes what is known about the structure of spherical (tomato bushy stunt virus, southern bean mosaic virus, satellite tobacco necrosis virus, other small RNA plant viruses, DNA plant viruses, bacteriophages, animal viruses) and helicoidal (tobacco mosaic virus, other helical plant viruses, filamentous bacteriophages) viruses as revealed by using the above methods.

Chapter 4 by M. H. V. van Regenmortel is a *par excellence* survey of serological methods in the identification and characterization of viruses. This is one of the most useful chapters to "methodology-minded" investigators who read this book for the sake of learning about methods s. str. The purpose of this chapter is celarly indicated in the introduction: it is meant "to present an integrated view of the various serological techniques that have been used in virology. The accent will be placed on the principles that govern each type of test and on the general applicability of the different serological techniques in all fields of virus research." A number of hints derived from first-hand experience help the investigator find the right way of doing things (preparation of the viral antigens, antisera, carrying out neutralization tests, agglutination tests, precipitation tests, test with labeled antibodies, complement fixation tests). In these descriptions a number of very up-to-date approaches, such as hemagglutination inhibition, latex test, various immunoelectrophoretic techniques, ELISA-test, radioimmuno assay etc. are also duely dealt with. A separate sub-chapter is devoted to immuno electron microscopy. The chapter concludes with a sub-chapter on the application of serological methods and an immense list of references.

Chapter 5 by H. Fraenkel-Conrat on the chemical modification of viruses is a lucid presentation of what is known about the effects of acylating agents, aldehydes, ketones, halogenating agents, alkylating and arylating agents, amines, bisulfite, imidoesters, nitrous acid, sulfhydryl reagents on the protein and/or nucleic acid moiety of viruses.

The two last chapters (Chapter 6 by T. M. Murphy and M. P. Gordon on the photobiology of RNA viruses and Chapter 7 by R. S. Day III on the photobiology of the DNA viruses λ, T4 and ΦX 174) differ very much from one another in their approach. Whereas the former one focuses on biochemical aspects that can be studied by using UV inactivation experiments, the latter is mostly concerned with genetic aspects. Although both chapters give us some insight into the principles of the mode of action of UV irradiation, and even (Chapter 7) laboratory UV sources are listed and principles of the evaluation of UV irradiation experiments presented, they both deal, from a theoretical point of view, with biochemical and genetic *results* obtained by using UV irradiation of viruses rather than methodology s. str.

This book, despite its heterogeneity with respect to the approaches different authors have used in writing their chapter, is useful in two ways: first, as a reference work that directs the expert to the proper source of a given method the details of which he happens to be concerned with, and second, as an introductory compendium for those who are interested in the principles of a certain method used in virus research. It is a pity that nucleic acid analytical methods (primary, secondary, tertiary structures) are not surveyed, even if just at the level of a condensed summary, in this valuable volume reflecting great efforts from the part of both the editors and contributors to integrate biophysical, biochemical and genetic methods that can be used in the study of viruses.

F. SOLYMOSY

Proliferation of Different Cell Types in the Brain by H. Korr. Advances in Anatomy, Embriology and Cell Biology, Vol. 61, Springer-Verlag, Berlin, Heidelberg, New York 1980, 72 p. with 21 figures

Shortly after the sites of nucleic acid synthesis were visualized in adult mouse brain using ^{14}C-labelled adenine and thymidine (Walker a. Leblond, 1958; Messier et al., 1958), in April, 1959, at the Annual Meeting of the American Anatomists, the first data on the localization of DNA synthesis in the embryonic nervous system were published simultaneously from two different laboratories (Sidman et al., 1959; Sidman a. Miale, 1959; Saurer, 1959) using ^{3}H-thymidine autoradiography.

Since the start of these pioneering works a vast number of papers have been published dealing with the proliferation of different cell types of the brain. This volume is the first survey in English on this topic reviewing 229 references. The other monographs (Gracheva N. C. The autoradiography of the nucleic acid and protein synthesis in the nervous system, "Nauka", Leningrad, 1968 and Reznhikov K. Yu. Cell proliferation in the vertebrate brain during normal development and after brain demages, "Nauka", Moscow, 1981) were published in Russian.

"The objective of the work — as stated by the author in the Introduction — was to answer the following questions concerning the proliferation of different cell types in the brain of untreated rats and mice during the course of pre- and postnatal ontogeny, based on the current state of knowledge:

1. In what period of ontogeny do the individual cell types or their precursores proliferate?
2. Which are the cell cycle parameters of these cell types?
3. What can be concluded about the mode of proliferation of the individual cell types?"

Although this work tends to summarize the modern concept of cell proliferation in the brain, it mainly concentrates on original contribution made in the author's laboratory. Experimental data are presented on the proliferative period of neurons, astrocytes, oligodendrocytes, cells of the subependymal layer and ependymal cells, epthelial, endothelial, meningeal cells, microglia and perycytes as well as cells of the choroid plexus. Cell-cycle parameters are calculated on the basis of different approaches, suitable for the study of proliferative processes in the brain.

The fact that the author is a well-known methodologist in the field of autoradiography having at the same time interest in brain development, may be one of the reasons why this work may attract interest.

As a whole this book provides a useful information for those who are interested both in the development of the rodent nervous system and in cell proliferation and its regulation.

Z. FÜLÖP

Membrane Biochemistry, A Laboratory Manual on Transport and Bioenergetics, by Carafoli, E. and Semenza, G. (editors), Springer Verlag, Berlin—Heidelberg—New York, 1979, 175 pages, 45 figures

This collection of methods written by a number of authors and compiled by two editors, contains 17 fundamental procedures and experimental arrangement, the programme of an advanced FEBS course held in Swiss Federal Institute during 1975—78. Following a brief introduction, each method is described in such a detailed form and with such accuracy that the volume reminds us of a collection of laboratory records. Consequently re-

production of these experiments should not be difficult. Obviously a tiny volume like this cannot aim at being all-inclusive. It has been written to help its readers solve some of the most frequently occurring experimental problems in this field.

Several chapters discuss the possibilities of assaying the transport of electrolytes (K^+, Na^+, Ca^{++}) and non-electrolytes across bacterial and mammalian membranes. Six sections deal with the examination of mitochondria, the preparation of vesiculi, analyses of redox processes, Ca^{++} transport, measurement of pH and determination of membrane potential.

For those interested in photophosphorilation three descriptions give basic information concerning materials of plant or bacterial origin. Finally, two chapters are concerned with the possibilities of using artificial lipid membranes and ionophores.

Some of the procedures described in the book are fairly simple, but some are more difficult, requiring expertise and fairly expensive equipment. The editorial policy is worth mentioning. Most respectably the editors intended to describe not only the techniques involved, but also to teach the reader to some degree how to design an experiment, how to put the proper question and what kind of answer to expect. In this respect the benefit of this book being multiauthorial is evident, as each author described his most favorite topic with expertise and enthusiasm.

P. Elődi

Bioorganic Chemistry, A Chemical Approach to the Enzyme Action by Herman Dugas and Christopher Penney, Series of Springer Advanced Texts in Chemistry, Charles R. Cantor, Editor. Springer Verlag New York, Heidelberg, Berlin, 1981, 508 p.

This textbook primarily serves teaching. It was not the authors' intention to cover all aspects of bioorganic chemistry. They presented a blend of general and selected topics to stress important aspects underlying the concepts of organic molecular model building.

The book contains seven chapters. After a short introduction (chapter 1), chapters 2 and 3 deal with the chemistry of amino acids and phosphates, respectively, including summaries of their biological synthesis. Chapter 4 treats the most important aspects of enzyme catalysis and discusses the mechanism of action of hydrolytic enzymes. Chapter 5 gives a good survey on enzyme models including micelles, polymers, cyclodextrins and the latest results on enzyme design. Chapter 6 is devoted to the role of metal ions in biological molecules. Thus zinc (carboxypeptidase A), iron (oxygen transport), copper (native proteins and models) and cobalt (vitamin B_{12}) are discussed. The final chapter deals with coenzyme chemistry involving oxidoreduction, pyridoxal phosphate, thiamine and biotin.

The references are by no means exhaustive. However, the reader can find additional references, since many of the citations are of books and review articles.

The book is a valuable source for students, teachers and other research workers who want to be acquainted with some of the selected topics of bioorganic chemistry.

L. Polgár

Muscle Contraction: Its Regulatory Mechanisms by Ebashi S., Maruyama K., and Endo M. (eds). Japan Sci. Soc. Press, Tokyo, Springer Verlag, Berlin, Heidelberg, New York, 1980 549 pages.

The book contains collected papers by the participants of a Symposium held in October 1979 in Japan.

One of the aims of the Symposium was to celebrate the seventy-fifth birthday of Prof. Hinski Kumagai and the twenty-fifth anniversary of Prof. Reiji Natori.

Prof. Kumagai organized the "Conference of the chemistry of muscular contraction", Tokyo in 1957, designed to open up relations with the West. The conference was the starting point of equalization in scientific achievement between Japan and the West. This was the first occasion after World War II to estimate their own level of achievement in the world.

Professor Natori developed a basically new tool for muscle research, namely the so-called "Natori's fibers" or skinned fibers.

The book consists of eight sections of special lectures as follows: "Cross-bridge Movement", "Dynamic Aspects of Thin Fila-

ments", "Troponin-linked Regulation", "Regulatory Mechanism in Smooth Muscle", "Myosin-linked Regulation", "Excitation–Contraction Coupling" and "Cytoskeleton".

One Special Lecture deals with the "Variety of Muscle Activating Systems" (F. A. Huxley), and the "Skinned Fiber, Past and Present" (Reiji Natori).

The titles of the main parts show that the 42 lectures include all fields of muscle biochemistry, physiology and biophysics and give an accurate account of present day research.

Some problems and conclusions drawn by the authors are of special interest and deserve proper attention. These will be enumerated as follows.

The time resolved low-angle X-ray diffraction studies on contracting muscle offer a possibility to understand the precise mechanism of force development (H. E. Huxley).

The myosin heads in heart muscle remaining near the thin filament during the diastolic phase may produce tension in the subsequent contraction more readily than those which have returned to the vicinity of the thick filament (Matsubara, I., et. al.).

It is reasonable to assume that the cycle *via* direct decomposition of AMpADP induces the rotation of myosin heads on the thin filament, which generates force (Arata, T.).

A specific sulfhydryl group located in the LMM region of myosin responsible for the activation of Mg^{2+}-ATP-ase of myosin B, brings about an increase in the affinity of myosin and actin in the myosin B system (Yamashita, T.).

The myosin attachment site is not located on the same side of actin groove as that of tropomyosin. This suggests that the movement of tropomyosin in the groove is not be the only important structural change during regulation (O'Brien, E. J. et al.).

Evidence bearing on the assignment of high affinity Ca^{2+} binding sites of troponin-C to regions III and IV and of low affinity sites to regions I and II is considered, particulary in the light of recent work on proteolytic fragments (Gergely, J., et al.).

The troponin system of striated muscle is a specialized form of the system that has evolved for the regulation of processes through changes in Ca^{2+} concentrations (Perry, S. V.).

The competitive inhibition of actomyosin ATP-ase by tropomyosin disappears when the myosin saturation of the actin filaments exceeds 5%. High degree of myosin saturation causes a complete shift of tropomyosin to the groove and the conformational change of all 7 actin molecules increasing their affinity for myosin (Murray, J. M. et al.). Immunoelectron microscopic study demonstrated that each of the three troponin components is distributed along the thin filament with a periodicity of 38 nm (Ohtsuki, I.).

A new model concerning the structure and function of regulatory proteins was proposed (Katayama, E.).

The possible interaction site of Tn–T (from cross linking reactions and specific chemical cleavages) is deduced as the region from 206 to 258 and the sites of TN–C as both N- and C-terminal sides (Ohara, O. et al.).

The myosin light chain kinase from chicken gizzard is known to consist of two subunits, and responsible for the initiation of contraction by phosphorylation of the 20 000-dalton light chains of myosin.

One subunit has been identified as calmodulin and the other contains the active site for transferase reaction. The stoichiometry of the two components is 1 : 1 (Hartshorne D. J. et al.).

Myosin light chain kinase can undergo reversible phosphorylation. cAMP-dependent protein kinase catalyzes the incorporation of 1 mol phosphate per mol of myosin kinase with a concomitant decrease of the binding of Ca^{2+} to myosin kinase as well as the activity. Dephosphorylation of myosin kinase by phosphatase results in an increase in kinase activity. A rise in the concentration of cAMP results in the relaxation of many different smooth muscles (Adelstein, R. S. et al.). Ebashi and colleagues favored an alternative view, namely, that phosphorylation is not required for smooth muscle actomyosin activation and a separate regulatory system is involved, called by them "leiotonin".

Addition of leiotonin complex to the actomyosin desensitized with tropomyosin activated the superprecipitation of this system in the presence of Ca^{2+} without phosphorylation of the "P" light chain (Nonomura, Y.).

Thin filaments from actin, tropomyosin and troponin of rabbit skeletal muscle cross-

linked in the presence of Ca^{2+} were always in the active "on" state, whereas those cross-linked in the absence of Ca^{2+} were always in the inactive "off" state. The thin filaments of smooth muscle treated with glutaraldehyde also froze in "on" and "off" states in activating the myosin ATP-ase (Mikawa, T.).

This result also confirms the Ebashi's concept about the smooth muscle actomyosin activation.

Calmodulin is considered in the regulation of smooth muscle as a multifunctional protein. Three forms of phosphodiesterase were isolated, only one of them could be activated by calmodulin (Head, J. F. et al.).

It is suggested that both regulatory and essential light chains are involved in myosin linked regulation and that only molecules retaining the subfragment 1—2 (S-1—S-2) hinge region are regulated (Szent-Györgyi A. G.).

It is suggested that the myosin-linked regulation rather than actin-linked regulation is operating effectively in squid myosin B (Konno, K. et al.).

In squid muscle, there is a dual regulation system (Tsuchiya, T. et al.). In frog skeletal muscle the Ca^{2+} release process is strongly voltage dependent (Chandler, W. K.).

The sarcoplasmic reticulum, even under normal conditions, enough amount of Ca^{2+} to produce maximum contraction of cardiac muscle (Kitazawa, T.).

It appears that connectin plays a role in keeping the A band at the center of a sarcomere (Maruiama, K.).

A new structural protein located in the Z line of chicken skeletal muscle was discovered. This 55 000 dalton protein formed lattice structure *in vitro* which were similar to those of the Z lines *in situ* (Ohashi, K. et al.).

Direct immunofluorescence microscopy of myofibrils showed that eu-actinin is localized at the Z line (Kuroda, M. et al.).

The book is well-documented, excellent for researchers in this field and the papers include an extensive list of references.

M. Bálint

Mechanics and Energetics of Biological Transport by E. Heinz, 1978. 35 Figures, 159 pages in *Molecular Biology, Biochemistry and Biophysics* Vol. 29 by A. Kleinzeller, G. F. Springler and H. G. Wittmann (eds)
Springer Verlag, Berlin, Heidelberg, New York.

Erich Heinz discusses biological transport basically in terms of the termodynamics of irreversible processes. It is of utmost importance to apply exact physical principles and methods in describing such complex physiological processes, since, at present, our knowledge is scanty concerning their molecular mechanism.

The author presents alternative models to describe certain transport processes, critically discussing the pros and cons of the individual models. Although the transport equations have been derived from different hypothetic models (because of didactical considerations) the resulting equations are relatively independent of these models and are given in the most generalized possible form.

As the author aims at describing the fundamental principles, he considers only relatively simple cases. In the discussed models at the most two kinds of flow, namely those of either two different solutes, or one solute plus one solvent, or heat, are studied. Owing to this great simplification the derived equations cannot always be used directly to solve a given biochemical problem, rather, the introduced principles and methods may serve as a good starting point towards the elaboration of calculations for sophisticated problems.

The book attempts to give an appropriate basis for studying the thermodynamics of transport processes. Accordingly, it introduces a few kinetic equations just to help the reader understand the thermodynamical relationships.

The leading principle of the monograph is that metabolism can only be described by considering a close interrelationship between the bioosmotic, i. e. the transport- and the biochemical processes.

The book consists of two major parts. The first is about the one flow systems — uncoupled transport with chapters on free diffusion, mechanism of interaction between solutes and problems of the irreversible thermodynamics of one flow systems. The second part contains the description of two flow systems-energetic coupling. In this part chapters on the special coupling of solutes as the

prerequisite of active transport, on the energetics of coupled transport and on the phase specific interactions, can be found.

The book is recommended to membrane biochemists, as well as to those researchers who are interested in the physical chemistry and biochemistry of biological transport.

I. SIMON

Basic Exercises in Immunochemistry by A. Nowotny A Laboratory Manual, (Second revised and enlarged edition) Springer Verlag, Berlin, Heidelberg, New York 1979. 314 pages.

This book is actually a textbook for students, devoted partly to those who wish to become familiar with the three main fields of immunochemistry and partly to those who need appropriate methods to solve immunochemical and immunbiological problems. The book consists of three parts: isolation of the substances, analysis of their chemical structure, and testing of their immunochemical and immunbiological activities.

The volume contains the fundamental methodology of immunochemistry introducing the most up-to-date methods for each topic. All this is outlined clearly, step by step, with utmost exactness in the form of 95 practical exercises. The author intended to write in such a style that anybody, with only marginal experimental and immunochemical background should be able to apply the increasingly difficult procedures, learn them and later use them as prescriptions.

In 32 practical exercises, extending to 98 pages, the book gives a fairly detailed description of protein purification methods in general, devoting particular attention to the immune proteins. Eight exercises introduce the fundamental preparation techniques, three the checking of these isolation steps (e. g. by electrophoresis, etc). Five exercises address themselves to the preparation of labelled proteins (with ^{125}I, DNP — sulfonic acid etc.), used for the various tests.

Sixteen chapters deal with the purification of substances important in other branches of immunology (antigens, agglutinins etc).

The following 32 exercises (on 112 pages) detail the different immunochemical applications of structural analyses. Of the qualitative analytical methods hydrolytic (three exercises), and chromatographic assays (seven exercises), amino acid analysis (two exercises), and carbohydrate determination (two exercises), are described. Quantitative assays are described for protein (four exercises), carbohydrate (11 exercises), and fatty acid (two exercises) determinations.

The third part of the book contains the immunological and biological tests (31 exercises, 98 pages). Two exercises are about antibody production, and four about agglutination. Ten exercises deal with different tests based on precipitation (immune diffusion, immunoelectrophoresis, radioimmunoassay, etc.). Ten oxidational exercises describe assays utilizing the effector functions of antibodies. Finally, five exercises deal with other immunobiological reactions.

Each exercise begins with a short description of the problem to be solved, followed by the enumeration of Materials and Equipment, with meticulous care. Then cover a very detailed description of the "Procedure", followed by the section "Evaluation" which gives invaluable help to the proper processing of the recorded data, or refers to other exercises. The section "Use and limitations" describes the applicability of the given method. Finally, a brief list of references, not intended to be complete, can be found at the end of each exercise.

Although not aimed at being comprehensive, the book still provides a fairly broad ground to both researchers and students wishing to acquire a deeper knowledge of immunochemistry and immunbiology.

62 figures contribute to a better understanding of the exercises.

F. KILÁR

Microtubules by P. Dustin. Springer-Verlag, Berlin Heidelberg New York 1978, 452 pages, 177 figures

The term microtubule was coined by Slautterback in 1963, therefore the experimental results of a relatively new topic of investigations have been reviewed in this book covering the literature predominantly of the period between 1966–1976. The significance of microtubules is underlined by the fact that

they are ubiquitous cellular structures (constructed in bulk through the assembly of specific protein building blocks, the tubulins) and that a variety of cellular functions are dependent on their integrity. Moreover, their biological importance is perhaps comparable with such cellular organelles as the ribosomes, the lysosomes or the mitochondria. Microtubule research is surveyed in this book by a recognized expert of the field who not only has prepared a comprehensive summary of the present state of knowledge and selected carefully from the vast and continuously expanding literature but added a thought-provoking outlook to perspectives of future investigations.

Following a brief introduction, the subject is discussed in 12 chapters. In a survey of the historical background the author shows how studies of the mitotic spindle poisons (first of all colchicine) led to the isolation of tubulin in the late sixties and emphasized that such poisons have been successfully used for the analysis of many cellular activities ever since. The chapters written on the structure, chemistry and general physiology of tubulins and microtubules demonstrate nicely the progress achieved in the last decade together with some fascinating problems that remained to be solved. These problems include the mechanism and regulation of assembly and disassembly of microtubules, the formation of various microtubule polymorphs, the roles of microtubule-associated proteins and the relations of microtubules to the cell membrane and various viruses. In these and later chapters alike, the multiple aspects of microtubule morphology are presented with adequate clarity, allowing the reader to gain extremely interesting insight into the supramolecular organization of these complex structures. This structural complexity is well illustrated by a thorough account of such highly ordered associations as the centrioles, the basal bodies, the cilia and flagella. A separate chapter has been devoted to microtubule poisons of which the parts written on colchicine and the Vinca alkaloids deserve special attention.

In five chapters the present knowledge on the role microtubules play in cell biology is surveyed. Although the phenomena treated in these chapters (the problems of cell shape, cellular movements, secretory processes, endo and exocytosis, neuroplasmic transport, mitosis etc.) are fairly diverse in nature, they have been brought together by microtubules serving as a common denominator. Furthermore, the recognition of the economic strategy of living systems to manage seemingly very different processes by means of the same basic mechanisms, coupled with the lucidity of the presentation, keeps the reader's attention focused on the frequently intricate details. Evidently, the high competence of the author is reflected to a great extent in these chapters reaching the top notch in the chapter on the mitotic process. The known relations of microtubule changes to various pathological states and the potency of colchicine to cure such inflammations as acute gouty arthritis justify that a special chapter on the medical and pathological aspects of microtubules has also been included.

No questions, this book will serve as a basis for future investigations on microtubules and further understanding of their biological functions. This volume is unique in the sense that no attempts to summarize microtubule science in such a comprehensive manner have been made previously. The value of this book is substantially augmented by the excellent quality of the photographs and schematic drawings which serve the purpose of both illustration and comprehension. Expectedly it will be an important source of references to cell biologists and research workers interested in microtubules and will be helpful in initiating the flow of new information and ideas into various textbooks of more general scopes. I can not help repeating a statement given by K. R. Porter in the Foreword to this volume: "it is to be hoped that Professor Dustin will find the time and enthusiasm to produce successive editions."

M. Tóth

Electron Microscopy at Molecular Dimensions (State of the Art and Strategies for the Future) by W. Baumeister and W. Vogell (eds) Springer V. Berlin, Heidelberg, New York) 1980

The Editors summarize the material of an international conference on the determination of the structure of biomacromolecules, held in the GFR in 1979. This book, published in the "Proceedings of Life Sciences" series

of Springer, contains 39 shorter chapters (in fact, published lectures) based on the work of 70 authors, whose postal addresses are also included.

These publications discuss mainly the authors' own new results, but the reference lists also allow a full review of the present state of the given special fields. (As a supplement, unfortunately only in one case, the presidential summary of the discussion of the special field is also included.)

The results are well documented: the book contains altogether 181 figures, mostly electron micrographs, the majority of which are of high quality, both technically and from the point of view of scientific information they convey.

The special fields covered by the book are the following: biological electron microscopy (including different techniques), molecular biology and its techniques and molecular structure. The subject of the book could be given essentially as "molecular microscopy", discussing not only present research but also future trends. "Molecular microscopy" is meant to be a specialized field of electron microscopy which, aiming at the determination of the structure of biological macromolecules and supramolecular organization, has made great progress in the last few years and will most probably play an important role in future molecular-biological research. However, in spite of the electron microscope being the main research tool in this field, one cannot agree with the way the discussion is limited to this one instrument, especially if the strategy for future research is also being considered. One must remember e. g. the dramatic progress of X-ray microscopy, the promising although more distant possibilities of neutron microscopy on the basis of the works of Australian and West-German scientists and even the topo-optical application of polarization microscopy cannot be disregarded as a supplementary method.

The first group of chapters discusses with many illustrations, included mainly the results so far obtained with a great variety of molecules. The subject of the second part covers the latest results in the field of image recording, low dose microscopy and image processing, as possibilities to overcome the present limitations set first of all by resolution and technical difficulties. The last 5 chapters discuss a problem of great practical importance: the investigation of the higher organization of ordered molecules, e. g. in membranes.

The detailed grouping of the chapters is the following: State of the Art (19 chapters), mainly morphological, electron diffraction and freeze-fracturing studies; Image Recording (5 chapters), TEM, SEM, STEM, image recoring, low dose image recording by TV techniques and the properties of photographic material for electron microscopy; Low Temperature Microscopy (3 chapters), the use of liquid helium, electron diffraction at low temperatures and decreasing radiation damage by the use of superconductive lens systems; Image Processing (7 chapters), reconstruction of the structure, including three-dimensional reconstruction. (It must be noted here that image processing is a rapidly developing field already with its own publications and conferences; the last international conference in this field was organized in Austria, at the University of Salzburg.) Artificial Assembly of 2-D Arraya (5 chapters), crystallization processes of biological macromolecules, aggregation of particles in membranes, assembling of proteins at lipid monolayers are the topics dealt with in further chapters.

A rapid review of the material of the conference is facilitated by a separate subject-index containing all the important key-words occurring in the book.

Our critical remarks mentioned above do not intend to diminish the main values of the book, which by all means is a publication of high standard, also supplying a great want. Thus one can only agree with the last sentence of the Editor's Introduction, saying that the aim of publishing the material of the conference in the form of a book was to help the wider community of "molecular microscopists" in solving those problems they face, for which an elaborated technique already exists.

This book will be a useful source of information to all research workers in the field of biology, biophysics, biochemistry and medicine, furthermore for instructors who, in the course of their experimental and theoretical work, study the physical structure of biomolecules; it may also be used as supplementary material in special postgraduate courses. Unfortunately, biologists and researchers working in similar fields rarely possess the

basic knowledge in crystallography etc. necessary techniques (electron diffraction, X-ray diffraction, analysis of electron contrast distribution, polarization photo-optics etc.).

On the other hand, biomineralization and the investigation of the physical structure of biomacromolecules are important, promising and developing fields of basic biological research. Therefore it is desirable and worth while that all the more researchers be familiar with this special field. Many of us who have participated in the biological application of structural studies and in the development of the field of biomineralization for a long time are especially content with this publication and highly recommend it to our younger colleagues.

I. VERES

Problems of Biological Physics, by L. A. Blumenfeld Springer-Verlag Berlin, Heidelberg, New York 1981

The seventh volume of the Springer Series in Synergetics, edited by H. Haken, has been written on the basis of a course "The Problems of Modern Biophysics" given by the author at the Moscow University School of Physics; it is valuable to a broad circle of readers who are interested in biophysical problems.

In this book some fundamental questions of biological physics are discussed. Most of them are different aspects of one of the most interesting biophysical problems viz. the relationship between statistics and mechanics in biological systems. Biological systems are considered as a priori entities developed during evolution. The author considers biophysics as a part of biology dealing with the physical aspects of the structure and function of certain comparatively simple biological systems, without considering their origin and evolution.

In the first part of the book the author analyzes a set of biophysical problems: the meaning of biological ordering, nonequilibrium thermodynamics in biology and statistical physics of biopolymers. In the remaining chapters the physical aspects of conformational and configurational changes of biopolymers, enzymatic catalysis, electron transfer and intracellular energy transformation are discussed.

Not only the problems that have already been more or less solved, or are to be solved are discussed, but the author points to those questions which he considers meaningless despite the vast literature that has been devoted to them.

The well-selected references also include interesting papers published in Russian which are not referred to in English papers and monographs.

Although this book is neither a textbook nor a monograph, it will provide students and scientists of biophysical sciences with a valuable source of information.

I. SIMON

Metal Ions in Biology Vol. 1. Nuclear Acid Metal Ion Interactions by T. G. Spiro (ed.) Wiley and Sons, New York, 1980. IX + 256 p.

This volume is the first in a new series "Metal Ions in Biology". The monographs in the volume emphasize the role of metal ions in biological systems and show how concepts from inorganic, coordination and structural chemistry can be applied to explain both the static and dynamic properties of metal complexes with biologically important ligands. This rather new field of science called inorganic biochemistry or bioinorganic chemistry attracted the attention of many inorganic chemists and biochemists and therefore a very rapid development can be experienced.

In the first volume the interactions of metal ions with nucleic acids are discussed.

Chapter 1 written by B. Rosenberg is entitled "Platinum Complexes for the Treatment of Cancer". The author gives a brief, but rather fascinating summary on the history of the discovery of platinum complexes of antitumour activity. Then he discusses the effects of platinum complexes on the bacterial cells and deals with the anticancer activity of these complexes drawing important general conclusions as to the activity of the most widely studied drug, the cis-dichlorodiammine-platinum (II). The questions are, how the molecular structure determines the anticancer activity, what the platinum complexes do to mammalian cells, how selective cancer destruction occurs, and how effective the platinum drugs against human cancer are. These are not only raised but also answered, on the

basis of information extracted from a variety of rather different types of investigation. The author concludes that "... it is now certain that combination therapies with the platinum drugs are a safe, effective treatment for a number of different types of cancer." (5 references).

The second review, by J. K. Barton and S. J. Lippard, has the title "Heavy Metal Interactions with Nucleic Acids". They begin with a discussion of metal binding sites on monomeric nucleic acid constituents and the complexes that form. Emphasis is laid on the selective heavy metal binding modes. Then they examine the metal binding to polynucleotides. The polynucleotide structure imposes conformational constraints which considerably influence the stereochemistry of the interactions. Also cases in which the metal ion serves as a probe of biological structure are discussed. The emphasis is centered on experiments that utilize the high electron density of the metal ion (e. g. electron microscopy, X-ray diffraction). Finally, proposals for the mode of action of cis-dichlorodiammineplatinum (II) are considered, and evidence from both *in vivo* and *in vitro* experiments, as well as proposed models for the specific geometry at the interaction site, are described. (219 references).

Chapter 3, "Metals and Genetic Miscoding", by L. A. Loch and R. A. Zakour, takes up the subject of metals as teratogens, mutagens and carcinogens. They consider the role of metals in the transfer of genetic information. The authors examine DNA synthesis *in vitro* to determine the effects of different metal ions on the fidelity of this synthesis, and then raise the question whether alterations in the fidelity of DNA synthesis are related to mutagenic and carcinogenic properties of metals. They conclude that mutagenic metal ions (e. g. Cr, Cu, Fe, Mn, Mo, Pt) after the fidelity of DNA synthesis. Correlations between alterations in fidelity *in vitro* and mutagenecity or carcinogenecity *in vivo* are also discussed. Finally the authors write about the mechanism of genetic miscoding by metals. (98 references).

The fourth chapter by M. M. Teeter, G. J. Quigley and A. Rich is on "Metal Ions and Transfer RNA", the one nucleic acid for which there are high-resolution three-dimensional crystal structures. The authors emphasize features of the metal binding sites that appear to be general for all tRNAs and then discuss the role these sites may play in tRNA and polynucleotide function. They consider the evidence for specific cation binding sites in tRNA (yeast), both from solution and crystallographic experiments, and discuss the binding site coordination and the relationship these sites may have to tRNA conformational stability. (53 references).

In Chapter 5 ("Structural Principles of Metal Ion-Nucleotide and Metal Ion-Nucleic Acid Interactions"), L. G. Marzilli, T. J. Kistenmacher and G. L. Eichhorn give a systematic discussion of the modes whereby metal ions may interact with the constituents of nucleic acids, as deduced from a variety of physical studies. They begin with a discussion of the relevant chemical and physical properties of nucleic acid constituents and write about metal binding modes and metal binding sites. Then they turn from the relationship between the structure of metal complexes of small molecules to the structure of metal complexes of polymers, and to the effects of metals on polynucleotide structure (e. g. conformational changes, cross-linking, unwinding, rewinding, catalytic action). Finally, the authors discuss some of the biological functions of metal ions (their role in DNA, RNA and protein synthesis, respectively). (227 references).

Volume 1 of this new series provides the reader with a wealth of novel information on the title subject. It can be highly recommended both to specialists and nonspecialists, since the chapters are readable accounts in a field that is both significant and under active research.

E. Kőrös

Metal Ions in Biology, Vol. 2. Metal Ion Activation of Dioxygen by T. G. Spiro (ed.) Wiley and Sons, New York, 1980, IX + 247 p.

This volume is the second in the series "Metal Ions in Biology" and focuses on how metal ions in biological systems activate molecular oxygen toward a variety of chemical reactions.

The first chapter, "O_2 Binding to Heme Proteins and Their Synthetic Analogs" by J. P. Collman, K. S. Suslick and Th. R. Halbert deals with the bioinorganic chemistry of myc•

globin and hemoglobin. The authors demonstrate how successful the synthetic analog approach to the study of metalloproteins is. In the first part of this review article they discuss the structure and function of O_2-carrying hemoproteins, then they turn to iron and cobalt porphyrins and to the axial coordination. This is followed by a detailed discussion of dioxygen binding to iron porphyrins. Here the authors describe how dioxygen complexes of Fe(II) can be stabilized. The principle approaches are low-temperature stabilization, attachment of porphyrin to a rigid polymer and construction of porphyrin ligands with steric constraints (picketfence porphyrins) that prevent bimolecular oxidation. Also the results of the kinetic and thermodynamic studies of O_2 binding are described. (209 references).

In the second chapter, "Cytochrome P-450 a Versatile Catalyst in Monooxygenation Reactions", M. J. Coon and R. E. White present an account of the important monooxygenase enzyme Cytochrome P-450 and indicate the probable mechanism by which it catalyses the dioxygen-dependent hydroxylation of a variety of substances. The authors emphasize the liver microsomal enzyme system because of its physiological importance, describe, however, also studies on this pigment from other sources. (175 references).

Chapter 3, "Mechanisms of Metal Catalyzed Oxygen Insertion", is written by J. T. Groves. The author discusses the mechanisms by which metal ions catalyze the insertion of O_2, and deals with the reactions of metal-peroxo and metal-oxo compounds, respectively, with alkenes. Then the chemical models of biological oxygen transfer, hydroxylations, respectively, and finally oxidations catalyzed by metalloporphyrins are considered. (98 references).

In Chapter 4, "Recent Progress on the Mechanism of Action of Dioxygenases", J. M. Wood discusses oxygenases, the non-heme iron enzymes that catalyze the insertion of molecular oxygen into organic substrates. He deals with the structure of dioxygenases, the substrate binding and kinetics, and gives a critical account of the dioxygenase mechanisms. (40 references).

Chapter 5, "Cytochrome c Oxidase" by B. G. Malmström describes the main properties of the rather complicated enzyme system that is responsible for the energy-coupled four-electron reduction of O_2 to H_2O in respiration. This outstanding account deals with the prosthetic group of the oxidase and of its substrate, with the structure of cytochrome c oxidase, with its spectroscopic, magnetic and oxidation–reduction properties, and with the catalytic reaction. Finally the author turns the readers' attention to yet unsolved problems. (105 references).

In the final chapter, "Superoxide, Superoxide Dismutases and Oxygen Toxicity" J. A. Fee gives an excellent critical analysis of the much-discussed role by superoxide and the metallosuperoxide dismutases in biology. The author tries to offer a unifying explanation of *in vitro* manifestations of superoxide toxicity, and discusses this and other aspects of the field of superoxide and superoxide dismutases which are relevant to the potential role of superoxide in oxygen toxicity. (240 references).

The contributions collected in this volume inform the reader on up-to day research and are written by distinguished authors of the respective fields. The chapters of this volume do not exhaust the subject of dioxygen and metal ions in biology, draw, however, the attention of both specialists and non-specialists to a rather exciting interdisciplinary field of research. This volume can also be highly recommended to life scientists, graduate students and coordination chemists.

E. KŐRÖS

Familial Hyperbilirubinemia by L. Okolicsanyi (ed.) John Wiley and Sons, Chichester—New York—Brisbane—Toronto, 1980

This excellent book contains the proceedings of the Workshop on Familial Disorders of Hepatic Bilirubin Metabolism. It lays special emphasis on Gilbert's syndrome and deals with the genetic, biochemical, and morphological aspects of this syndrome and the other genetically determined hyperbilirubinemias. It discusses in detail the metabolism of the various organic anions in these conditions and describes clinico-pharmacological examinations with a diagnostic or differential diagnostic purpose. The book may be of interest to gastroenterologists, hematologists, pediatricians, geneticists, clinical biochemists,

pharmacologists, and physiologists. It is the work of 61 senior authors, in the majority of cases with coauthors, who are internationally acknowledged experts of this special field (35 Italians, 5 Americans and Belgians, 4 Frenchmen and Austrians, 2 Englishmen, Swiss, GFR and Japanese).

B. H. Billing (Dept. of Med., Royal Free Hosp., London) surveys the groups of familial unconjugated hyperbilirubinemias, their differential diagnostic criteria, their clinical and biochemical aspects, setting apart the Crigler-Najjar type I and type II syndromes. In the first, the defect of UDP glucuronyltransferase (UDP-GT) is complete; the patients do not respond to the microsomal enzyme inductors (as against type II, in which partial enzyme activity is detectable). The author emphasizes that the reduced activity of UDP-GT occurs in many syndromes, thus also in chronic persistent hepatitis, Wilson's disease, and hemolytic diseases. The decrease of UDP-GT in G.'s syndrome is variable; most often, it is manifested in the hemolytic stress effects, with decreased bilirubin. The author mentions as a diagnostic test, as a nonspecific test for G.'s syndrome, the significant rise in serum bilirubin induced by fasting and nicotinic acid, as well as abnormal bilirubin kinetics. In 50% of the patients with G.'s syndrome, the survival of the red blood cells was reduced. Marked hypertrophy of the smooth endoplasmic reticulum was observed in G.'s syndrome.

P. Beck (Mount Sinai School of Med. Hematol. Center, N. Y.) studied the distribution of the serum bilirubin levels in human material. He discusses the value of isotopic liver function tests, among them that of *hepatic bilirubin clearance* (C_{BR}) and that of *plasma bilirubin turnover* (BRT).

The elevated level of direct bilirubin is a marked sensitive and virtually specific test of hepatobiliary dysfunction. Berk and co-workers demonstrated reduction of the C_{BR} to one third of the normal value. Thus, C_{BR} has a differential diagnostic importance, as its value is normal in hemolytic conditions. The authors doubt the specific diagnostic value of C_{BR} because they found it decreased in the preoperative phase also in their hereditary spherocytetic patients.

Extreme lipofuscin storage in the liver biopsy specimen is characteristic in G.'s syndrome, and its connection with abnormal bilirubin transport is still doubtful. According to Bailey and co-workers, G.'s syndrome is a distinct genetic entity, which affects about 2% of the population, and they consider it proven by pedigree analyses that the Criggler-Najjar syndrome type II represents a homozygous state for the Gilbert syndrome gene (Hunter and coworkers). J. Fevery and co-workers (Lab. Hepat., Hematol., Leuven) summarize the differential diagnosis and the biochemical aspects of chronic unconjugated hyperbilirubinemia (CNHB); describing the forms of dyserythropoiesis, they discuss the morphological differential diagnostic value of the bone marrow in congenital dyserythropoietic anemias and analyze the differential diagnosis of the hemolytic conditions. They present a list of modern methods, among the *H^3-thymidine incorporation* in circulating erythrocytes, *^{15}C-glycine incorporation*, *^{59}Fe incorporation* into the red cell hemoglobin, and the *bilirubin production rate*. On the basis of the plasma clearance of *bilirubin* or *isotope-labeled bilirubin* (Billing and co-workers), impairment of the bilirubin turnover can be demonstrated; the same is line of exogenous bilirubin loading in G.'s syndrome.

Nicotinic acid-induced hyperbilirubinemia (Fromke and Miller) is diagnostically advisable in G.'s syndrome and can be regarded as an endogenous bilirubin loading test. *^{51}Cr-labeled erythrocyte survival* and demonstration of bilirubin conjugates in the bile give further information for the differential diagnosis as also the *starvation-induced hyperbilirubinemia test*.

Okolicsanyi and co-workers (1980) reported that the starvation test made correct diagnosis possible in 40% of their patients, as against 100% of the 4-hour plasma retention value after bilirubin loading.

Determination of the rate of bilirubin reflux and hepatic storage is an important source of information.

C. Tiribelli and co-workers (Inst. Pat. Med. Triest) studied the possible role of bilitranslocase (BTL) in taffy acid transport.

N. Blacnkaert (Liver Center, Dept. Med. Univ. Calif., San Francisco) detected disturbance of bilirubin conjugation in *Criggler-Najjar disease* and in *Gunn rats*. The Criggler-Najjar disease (C—N) is a chronic non-hemolytic indirect hyperbilirubinemia; its type I is

characterized by severe bilirubin encephalopathy, phenobarbital resistance of the hyperbilirubinemia, and colorless bile, containing bilirubin only in traces, while in C—N type II indirect hyperbilirubinemia is less severe (below 340 μmol/l = 20 mg/100 ml), is unaccompanied by encephalopathy, but phenobarbital sensitivity is present. Hepatic ZDP-GT activity is zero or minimal in both groups. The typical form of inheritance of type I is autosomal, recessive, that of type II autosomal, dominant. The clearance rate of ^{14}C and ^{3}H bilirubin indicated disturbance of the endogenous bilirubin metabolism beside normal BRT and markedly decreased CBR.

The discussion of the biochemical and ultrastructural localization of the mechanism of bilirubin conjugation is excellent.

Muraca and co-workers (Lab. Hepat. Leuven) studied the influence of sex on bilirubin excretion in rats and demonstrated that UDP-GT activity is higher in female than in male rats. Human studies show similar results.

A. M. Jezequel and co-workers (Cell Pharm. Dig. dist. Unit., Dept. Gastroent. Acona) surveying the *ultrastructure of unconjugated hyperbilirubinemias* observed that in G.'s syndrome there was a significant increase in the surface density of the rough endoplasmic reticulum, the mitochondrial membrane, and the cristae.

Surrenti (III. Med. Path. Gastroent. Univ. Florence) reported on primary shunt hyperbilirubinemia.

R. Schmid, serving as moderator (Liver Center, Univ. Calif., San Francisco) described familial disorders of hepatic bilirubin metabolism.

R. P. H. Thompson's (St. Thomas' Hosp. London) article deals with the *genetic transmission of G.'s syndrome.*

Baroody and Shugart described dominant inheritance with incomplete manifestation in G.'s syndrome. Menthol loading proved adequate to demonstrate the gene carriers. The conjugation defect suggested dominant genetic transmission with varying expressivity.

The chapter written by B. F. Scharschmidt (Univ. Calif., San Francisco) deals with *bilirubin kinetics in G.'s syndrome* and their clinical and pathological aspects. The decrease of C_{BR} in G.'s syndrome has been proved. Hepatic bilirubin uptake and storage may also decrease, but the latter defect is possibly due to UDP-GT defect. The author discusses in detail the informative value of the tests for G.'s syndrome. In his opinion the bilirubin response to nicotine amide and to fasting is specific, BSP clearance and ICG clearance are non-sensitive and non-specific, C_{BR} and UDP-GT are sensitive, but non-specific.

E. Ventura and co-workers (Univ. di Modena I. Padova, Verona) in their multicenter study discuss the results of *crystalline bilirubin kinetics* in G.'s syndrome.

C. Cobelli and co-workers (Inst. Electrotechn. Univ. Padova) also dealt with bilirubin kinetics and the diagnostic value of BSP in G.'s syndrome.

J. L. Gollan (Univ. Calif.) summed up the mechanism of fasting- and diet-induced hyperbilirubinemia,proving that fasting-induced hyperbilirubinemia is more severe in the case of congenital absence of UDP-GT activity, thus in Gunn rats, in G.'s syndrome, and C—N II syndrome as controls.

J. M. Metreau (Hop. Henri Mondor, Fr.) and co-worker deal with the *definition and pathogenesis of G.'s syndrome.*

K. Okuda and co-worker (I. Dept. Med., Chiba Univ. Japan) review the studies of organic anion kinetics in patients with G.'s syndrome. They distinguish two groups on the basis of the kinetics of indocyanine green (ICG) and BSP plasma clearance.

B. Bircher (Dept. Clin. Pharm. Univ. Bern) investigated the matabolism of various drugs in G.'s syndrome. He demonstrated that in G.'s syndrome increased drug toxicity generally does not occur. Besides sulfobromophthalein, rifamycin-SV, menthol and aminopyrine, he proposed tolbutamide as test compound for the diagnosis of G.'s syndrome.

J. Bircher and co-workers studied aminopyrine and sulfadimidine acetylation in G.'s syndrome and found that the distribution of the acetylator phenotypes differs from that observed in the control population; thus a genetic correlation between G.'s syndrome and the acetylator status is probable.

M. Venuti and co-workers (I. Clin. Med. Univ. Padova) studied *antipyrine and galactose kinetics* in patients with G.'s syndrome. They found that the primary metabolism or

half-life of these substances in the liver was the same as in the controls, i. e. the pharmacometabolic activity of the liver was not impaired in G.'s syndrome. On the basis of the scattering of antipyrine half-life, the authors suppose clinical heterogeneity of this syndrome.

F. Lirussi and co-workers (Univ. Padova) studied bile acid kinetics in Gilbert syndrome patients and demonstrated with the help of 24-14-C-Cholic acid and Chenodeoxycholic acid kinetics that the bile acid transport was unaffected.

M. Coltorti and co-workers (Inst. Semeiotica Med. Naples) report prolonged half-lives of *nicotinic acid and rifamycin-SV* in G.'syndrome, underlining that pretreatment with phenobarbital significantly reduces the effect of these drugs.

Kutz and co-workers (Med. Polikl. Univ. Bonn) demonstrated impairment of the conjugation of chlorophenoxyisobutyric acid in G.'s syndrome.

N. Carulli and co-workers (Inst. Clin. Med. Univ. di Modeno) discuss the diagnostic value of the tolbutamide retention test for the diagnosis of G.'s syndrome. In their opinion it has diagnostic value and is suitable for differentiating 3 subtypes of G.'s syndrome.

A. V. Wolkoff (Liver Res. Centr. Bronx) in his article deals with *chronic conjugated hyperbilirubinemias*, thus with the Dubin-Johnson and Rotor's syndromes. The author calls attention to the normal histological picture of the liver structure as revealed by light microscopy and the storage of a melanine-like pigment in the hepatocytes. The isomers coproporphyrin I and III can normally be demonstrated in the urine. 75% of them is discharged as coproporphyrin III in the urine. In the Dubin-Johnson syndrome the total excretion is normal, but that of coproporphyrin I is above 80%. In obligate heterozygotes the total coproporphyrin excretion is reduced by about 40%, coproporphyrin is reduced by 50%, coproporhyrin I is increased, between the levels of the normal and the homozygotes. Therefore, this syndrome is considered to be a disturbance of porphyrin biosynthesis. This hypothesis, however, is not yet proven. Its inheritance was found to be an autosomal recessive characteristic.

The list of pertinent literature at the end of each chapter is complete, comprehensive, and upto-date. Okolicsanyi's work of editing deserves the highest praise, the structure of the book is uniform.

A. László

Platelets: Cellular Response, Mechanisms and their Biological Significance by A. Rotman, F. A. Mayer, and A. Silberger (editors) John Wiley et Sons, Chichester, New York, Brisbane, Toronto, 1980. 73 Figures, 38 Tables

The book includes lectures of an EMBO Workshop held in the Weizmann Institute of Science (Rehovot, Israel) from 14 to 16 April, 1980. The 22 lectures cover four broad fields and discuss cellular and molecular events that take place during thrombocyte activation.

Under the title "Platelet Function" lectures are presented on the role of thrombocytes in blood arrest, on the relationships between the streaming of blood and thrombocyte aggregation, on those between the ADP content of erythrocytes and thrombocyte adhesion on the reactivity of injured endothelial cells, on the structure and haemostatic function of collagen, as well as on the role of calcium in thrombocyte reactions.

The second part bears the title "Platelet membrane structure and receptors". Although the plasma membrane of thrombocytes shows a close morphological resemblance to that of other cells, yet, it is unique in the sense that numerous reactions of thrombocytes are membrane associated or connected with processes of membrane origin. In this respect membrane glycoproteins seem to have an exceptional importance. Several lectures discuss their role in the functions of thrombocytes, in aggregation and in binding of thrombin and ADP. In addition lectures on the isolation of membrane glycoproteins, on the lipid–protein interactions formed in the membrane during activation and on the role of fibrinogen during aggregation, are presented here.

In the chapter "Intracellular Platelet Response" lectures on the roleand frame-stabilizing function of microtubuli and microfilaments of thrombocytes, on the synthesis of prostaglandin in thrombocytes on the effect of anti-aggregation agents, as well as papers dealing with the mechanism of release reaction are of interest.

Finally, in the chapter "Platelet Pharmacology" the bearing of this multiple problem on the biochemistry of biogenic amines is summarized.

As thrombocytes resemble in many ways to neurons, their application as model cells is remarkable. In this respect reports dealing with the uptake, storage and release of serotonine, as well as with pharmaceutical modifying these functions, deserve special attention. Also, papers discussing changes in the activity of monoaminooxidase of thrombocytes observed in schizophrenic patients, are of particular interest.

S. Elődi

Immobilized Enzymes. An introduction and applications in biotechnology. by Michael D. Trevan, John Wiley and Sons, Chichester–New York–Brisbane–Toronto, 1980. pp. 138.

This book deals with various aspects of enzyme immobilization and immobilized enzymes. It is divided into five chapters.

The first chapter gives a broad outline of the different immobilization techniques and describes the main points how to choose a particular immobilization technique.

The second chapter is a survey of the effects immobilization may have on the enzyme molecule itself and on its kinetic parameters. This chapter is subdivided into four parts dealing with (1) the microenvironment, (2) the effects on enzyme molecules, (3) perturbation of enzyme pH dependence, and (4) effective kinetic parameters.

Chapter 3 is devoted to the possible and already existing applications of immobilized enzymes, including analytical, therapeutic, industrial and preparative uses. Just to pick out some of the topics mentioned: glucose-sensitive electrode; enzyme replacement; resolution of DL-amino acids; production of high fructose syrups; treatment of milk; economic and practical aspects, etc. The last part of this chapter attempts to predict some future trends in the field (coenzyme recycling, conversion of hydrophobic substrates, multistep synthesis, waste utilization, processes concerned with energy production).

Chapter 4 discusses the information we can get from studies of immobilized enzymes set up to model biological systems. The sections of this chapter are the following: modifications of enzyme reactions by mechanical stress, model multienzyme systems, models of active transport, heterogenesis (model systems which generate or utilize heterogeneity — current generation, hysteresis, memory, oscillations). The final section points to the biological relevance of model immobilized enzyme systems.

Chapter 5 is a brief cookery-book for those who took a fancy to make some immobilized enzyme preparations with their own hands.

The author promises in the preface to provide an introductory text to the fascinating subject of immobilized enzymes. He promises to omit unnecessary and confusing details and keep explanation as unmathematical as possible. Indeed, he keeps his promise. The referee, who works in this field, sometimes felt that not only unnecessary and confusing but even important details are missing. Nevertheless, it is stated that the book is written for "non-expert" readers, and thus the clear style, the bright logic of the book and the fact that the author gets at the heart of the topic in question, amply compensate for this feeling of want. Thus the book is very useful to all those who want to inquire about the main features, possibilities and limits of this multidisciplinary field, and helps the beginner find the way in the huge and heterogeneous literature.

Veronika Jancsik

The Physical Behaviour of Macromolecules with Biological Function by S. P. Spragg. In: Monographs in Molecular Biophysics and Biochemistry, edited by H. Gutfreund, 202 pages with 35 figures, John Wiley et Sons, Chichester— New York— Brisbane— Toronto 1980

This is a rather unorthodox monograph on the solution properties of biological macromolecules. The experimental work presented and discussed is mainly related to proteins and their interactions with similar solute molecules and with the solvent. The basic concept is that an aqueous solution of biological macromolecules must be considered as a whole unit. The author's aim is to relate experimental results and theory and to explain how the detailed structural features modify the organization and activity of the solvent.

This book is written to the biochemist and the biophysical chemist and only moderate knowledge of mathematics and physics is required to understand the concept. However, some experience in theoretical and experimental physical chemistry is required to follow the text. The main emphasis is on the detailed treatment of the concentration dependence of hydrodynamic parameters in terms of long range interactions between solute molecules, and the biological significance of these interactions is stressed. The importance of week energies in regulating macromolecular systems in biology is also emphasized, and dynamic aspects of the macromolecular conformation are analyzed in detail. In the monograph existing theories are applied to specific problems, the approach however, is new.

The book is divided into four chapters. Chapter 1 gives a summary of the physicochemical concepts used in the specific chapters which follow. General properties of aqueous solutions are briefly summarized and thermodynamic implications, chemical equilibrium are described. There is a concise paragraph about molecular forces and energies including ionic forces, hydrogen bond, dipole interactions and van der Waal's forces.

Chapter 2 is a critical survey of methods for estimating molecular masses, and includes the measurement of osmotic pressure, intensity light scattering and ultracentrifugation. Gel-chromatography and gel-electrophoresis are also discussed. The conclusions are summarized at the end of the chapter in a separate paragraph calling attention to the purity of the samples, interactions with small ions, concentration dependence, calibration of the method, and to the informative values of differential techniques.

In chapter 3 dynamic aspects of macromolecular structure are outlined. The chapter is a fortunate alloy of experimental and theoretical treatments. Diffusion and flexing are scrutinized and structural and functional aspects are analyzed together. According to the author's personal interest expertise diffusion and quasi-elastic light scattering are discussed in more detail than other questions.

Quasi-elastic light scattering is chosen to study the effect of small changes in molecular structure on the motions of the macromolecule. This chapter is also centered about long-ranging weak forces and their contribution to the functional behaviour of biological macromolecules.

A separate chapter — chapter 4 — is devoted to the behaviour of sticky molecules. It includes a summary of mathematical models of specific macromolecular interactions, and of the experimental methods to estimate interaction parameters. Specific examples of strong, medium and weak interactions are presented. A paragraph is devoted to dynamic relaxation as a tool to study macromolecular interactions by perturbing the equilibrium condition and monitoring the relaxation back to equilibrium. The question of underlying principles of the evolution of multisubunit enzymes is also raised.

This book — despite its pessimistic tone — can be suggested as a useful reading to every biochemist or biophysicist working on macromolecules. The aim of this monograph is to interpret macromolecular behaviour and function on the basis of molecular details and physical interactions. It is not the authors fault that the picture is not always clear, since our present knowledge is not sufficient to give an exact physical treatment of macromolecular interactions. Spragg's book is a successful attempt to guide the physical chemist where physical reality ends and speculation begins, and it gives a profound qualitative picture about the complexity of macromolecular interactions.

P. ZÁVODSZKY

Cell-hybridization and Mutagenesis of Somatic Cells of Animal- and Vegetable Origin in vitro in: Ergebnisse der experimentellen Medizin (Vol. 34) by J. Schöneich (ed.). VEB Verlag Volk und Gesundheit, Berlin, 1979

In this volume 16 communications have been published. Of the *in vitro* methods of examination which appear to be the basic method used throughout the communications, the most various methods of cell and tissue-breeding were applied, e. g. cell culture prepared from human spleen was used by K. H. Grzeschik for the further cytogenetic and biochemical analysis. In the course of breeding animal tissues intraspecies somatic cell-hybrides with chromosomal instability were produced (J. Raskó et al.). In other experi-

ments (Vera Spurna and M. Nebola, CSSR) a somatic cell model was produced by the fusion of malignant and non-malignant cells. *In vitro* developing spontaneous mamillary neoplasms have been examined (Renate Vidmaier and Gisela Papsdorf, GDR). Frequency of mutagenesis due to the effect of the oncogenic Simian Virus 40 (SV 40) was examined in 3T3 fibroblast cells of mice (M. Theile et al., GDR). Physiological characteristics of isolated protoplasts of higher plants were examined by H. Koblitz (GDR) for purposes of somatic cell genetics; A. J. Müller (GDR) summarized the results so far obtained, and the further perspectives, in the field of somatic cell hybridization of higher plants. Somatic compatibility was examined after fusion of plant protoplasts (Dudits et al., Hungary). KR 103 cell-line of *Nicotiana sylvestris* was examined in a suspension culture (P. Maliga et al., Hungary). Cytoblast fusion is described in the case of *Nicotiana sylvestris* and *Nicotiana knightiana* (L. Menczel et al., Hungary). Defect in nitrate reductase of cells of higher plants is the selective marker of somatic hybridization in Grofe's (GDR) investigations. Biochemical characteristics of the nitrate reductase-deficient Nicotiana cell line are explored by R. Mendel (GDR). N-nitroso-N-methyl-carbamide-NMK effect of mutageneous substances was investigated by S. L. Karanova et al. (USSR) in cell cultures of *Dioscorea deltoidea*.

Tomato cells have been used as cell models (Z. Opatrny, CSSR). Cytological and karyological examinations of cell cultures of the barley led E. U. Scheuhert (GDR) to the conclusion that, in barley cell cultures, the intensive callus formation is accompanied by cytological instability. G. Saalboch observed an organotypic increase of callus culture upon the effect of treatment with extracts made from seedlings of different barley species. Experiments on cell hybridization added much to the detailed analysis of various processes of differentiation and gave important results in both biological and biochemical aspects. Such investigations are of great importance also in different areas of oncological research.

Remarkable results have recently been achieved in connection with the induction and mutation phenomena observable *in vitro*.

The large-spectrum and up-to-date presentation of the *in vitro* methods of examination make the volume highly recommandable not only to experimental biologists, biochemists, physiologists, but also to practitioners interested in genetics or working in this field.

Esther KAPA

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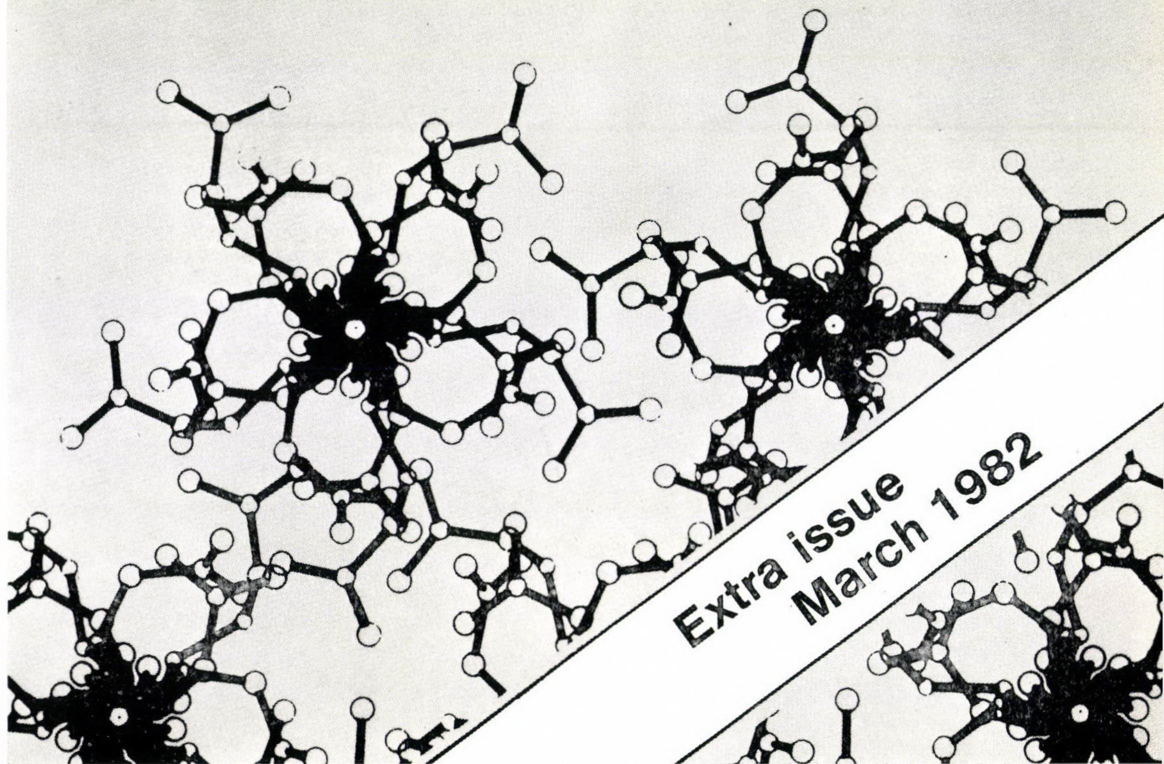

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