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Effect of Ultraviolet Irradiation on the Quaternary Structure of Rabbit Muscle Aldolase

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(Received October 20, 1972)

The effect of UV irradiation on rabbit muscle aldolase was investigated. Deviation of inactivation rate constants from the theoretical values below 1 mg/ml protein concentration indicated that UV light induced the dissociation of the tetrameric aldolase. Dissociation was proved by the hybridization test.

Introduction

Rabbit muscle aldolase (fructose 1,6-diphosphate: D-glyceraldehyde 3-phosphate lyase, EC 4.1.2.13) is an oligomeric enzyme (mol. wt.: 158 000) (Kawahara, Tanford, 1966; Závodszky, Biszku, 1967) consisting of four nearly identical subunits (Sajgó, 1971; Lai, 1968) each bearing one active center (Penhoet et al., 1966; Meighen, Schachman, 1970). Most pertinent data suggest that aldolase is a very stable tetramer, i.e., it dissociates into subunits only on drastic effects, such as acid or urea treatment.

We undertook to study the possible spontaneous dissociation of the tetrameric enzyme in an indirect way. Our approach was based on the reasonable assumption that the different aggregational forms exhibit different sensitivities to damaging effects. The heat denaturation of aldolase (Friedrich et al., 1972) suggested that the enzyme does not dissociate into dimers or monomers down to about 10 μg/ml protein concentration.

Ultraviolet light has been reported to inactivate a number of enzymes including aldolase (Setlow, Boyle, 1957). In the present work we studied the mechanism of UV-induced inactivation of the enzyme. We found that UV irradiation at 25° rapidly inactivated aldolase; the rate of inactivation below 1 mg/ml protein concentration was enhanced due probably to the induced dissociation of the tetramer followed by the rapid denaturation of monomers.

Materials and methods

Rabbit muscle aldolase was prepared and three times recrystallized by the method of Taylor et al. (1948). Protein concentration of aldolase solutions was determined spectrophotometrically at 280 nm by using the absorption coefficient

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$A_{280}^{0.1\%} = 0.74$ (Biszku et al., 1964). Enzyme activity was assayed by the dinitrophenylhydrazine method of Swenson and Boyer (1957) as described earlier (Friedrich et al., 1972). Succinyl aldolase for the hybridization experiments was prepared according to Meighen and Schachman (1970) by using 2.8 moles of succinic anhydride per mole lysine (calculated with 25 lysyl residues per aldolase subunit).

Irradiation with UV light was carried out in 50 ml beakers merged into a $25 \pm 0.2°$ water bath. A 250 W low pressure mercury lamp positioned at 58 cm distance above the surface of aldolase solution served as light source. In the standard setup 2.8 ml samples of aldolase in 0.2M Tris · HCl buffer, pH 7.5, were irradiated; surface area: 9.1 sq. cm; solution column height: 0.22 cm. Frequent stirring of the sample did not alter the time course of inactivation. For each experimental point a separate sample was used.

Polyacrylamide gel-electrophoresis was performed as described by Hedrick and Smith (1968) at pH 8.5, in a Reanal Modell-69 type disc-electrophoretic apparatus. The gels were evaluated densitometrically.

Results and discussion

1. Time course of inactivation of aldolase on UV irradiation

Irradiation with UV light caused the rapid inactivation of aldolase (Fig. 1). The inactivation followed first-order kinetics to about 5% residual activity.

To be able to predict the rate of inactivation at different enzyme concentrations, the absorption coefficient for the inactivating radiation should be known. As we worked with non-monochromatic light, the coefficient was estimated experimentally on the basis of the following approximation.

In case of surface irradiation the time course of inactivation is described by the equation

$$A = A_0 e^{-\alpha I t}, \tag{1}$$

where A_0 = initial enzyme activity, A = enzyme activity at time t, α = sensitivity parameter, I = intensity of incident light, and t = time. Thus $\alpha I = k$, the apparent first-order rate constant of inactivation.

If a layer of thickness l is irradiated, one can calculate in a first approximation with an average intensity,

$$I = \frac{1}{l} \int_o^l I_0 e^{-\beta c l} dl, \tag{2}$$

where c = concentration of aldolase, and β = absorption coefficient of aldolase at the critical wave length(s). Since at constant layer thickness $\beta l = b$, it can be

shown that the integrated form of equation (2) substituted into the expression of k yields

$$k = \alpha I_0 \frac{1 - e^{-bc}}{bc} . \tag{3}$$

The plot k *vs.* $\frac{1 - e^{-bc}}{bc}$ should give a straight line if the sensitivity parameter α is independent of protein concentration.

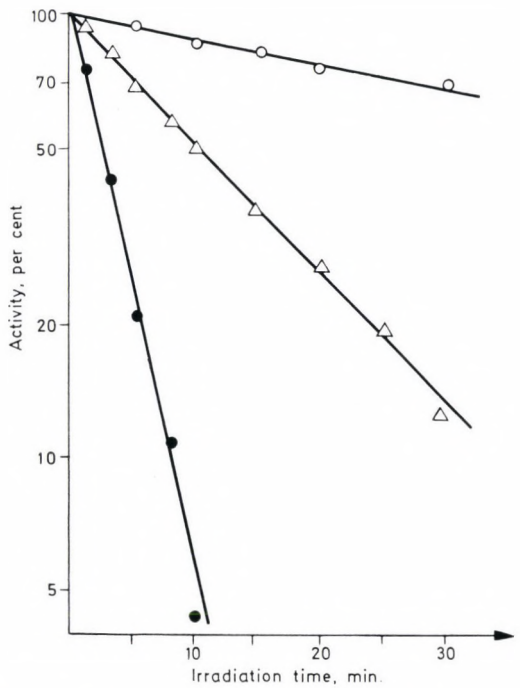

Fig. 1. Time course of inactivation of aldolase by UV irradiation. Aldolase concentrations, ○—○: 20 mg/ml; △—△: 1 mg/ml; ●—●: 0.01 mg/ml in 0.2 M Tris · HCl buffer, pH 7.5, at 25°C

We measured k as a function of aldolase concentration in the 1 to 20 mg/ml range, where the enzyme is known to exist as a tetramer. Then the value of b was selected by trial and error with which the experimental points gave the best fit to a straight line intersecting the origin. This value was found to be $b = 5 \times 10^4\ M^{-1}$, when $\alpha I_0 = 7.8 \times 10^{-2}\ min^{-1}$ (Fig. 2). The fact that the points fit well to a straight line suggests that the sensitivity parameter is indeed independent of protein concentration in the range examined.

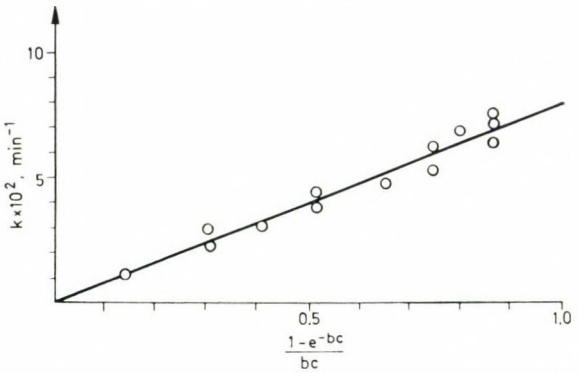

Fig. 2. Plot of equation (3) for the estimation of absorption coefficient; cf. text

2. Dependence of the rate of UV-induced inactivation on protein concentration

Fig. 3 shows the rate constant of UV inactivation of aldolase as a function of enzyme concentration over the 0.005 to 20.0 mg/ml range. The solid line is the theoretical curve calculated from equation (3) with the constants given above. Apparently, below 1 mg/ml concentration the measured values of k increasingly deviated from the predicted ones. However, if irradiation is carried out in the presence of 1 mg/ml serum albumin, the enhancement of k values towards low aldolase concentration does not occur.

Apparently, in the absence of serum albumin the mechanism of UV-induced inactivation is changed around 1 mg/ml aldolase concentration. One of the possible reasons for the greater susceptibility would be the spontaneous dissociation of tetrameric aldolase into lower aggregational forms. However, this explanation seems improbable on the following ground: *a)* deviation from the expected values commences at relatively high aldolase concentration, 1 mg/ml; spontaneous dissociation of such an extent could not have escaped detection by other methods, *b)* serum albumin prevents the increase in the rate of inactivation, whereas a true chemical equilibrium should not be affected by the presence of a foreign protein.

The observed deviation from the expected behaviour, as shown in Fig. 3, might then be traced back to two alternative reasons as follows.

a) Equation (3) does not satisfactorily describe the process of inactivation below 1 mg/ml aldolase concentration. If we assume that there is more than one critical wave-length, i.e., if inactivation is a complex result of the action of photons of different energies, then practically any kind of theoretical curve can be constructed by extending equation (3) and by adopting appropriate constants. Indeed, a curve fitting to the experimental points (without serum albumin) in Fig. 3 can be obtained if we assume that in addition to the inactivating light of parameters α and β, there is another one of parameters α' and β', where $\alpha \ll \alpha'$ and $\beta \ll \beta'$ the effect of which can only be noticed at low protein concentration.

b) Alternatively, if inactivation is mainly due to photons of the same energy, the deviation in Fig. 3 can be explained in terms of an induced dissociation-recombination mechanism analogous to that described by Südi and Khan (1970) for the heat inactivation of lactate dehydrogenase. Accordingly, as a subunit in the aldolase tetramer absorbed the critical amount of photons, it is denatured with the concomitant break-up of all intersubunit bonds within the tetramer.

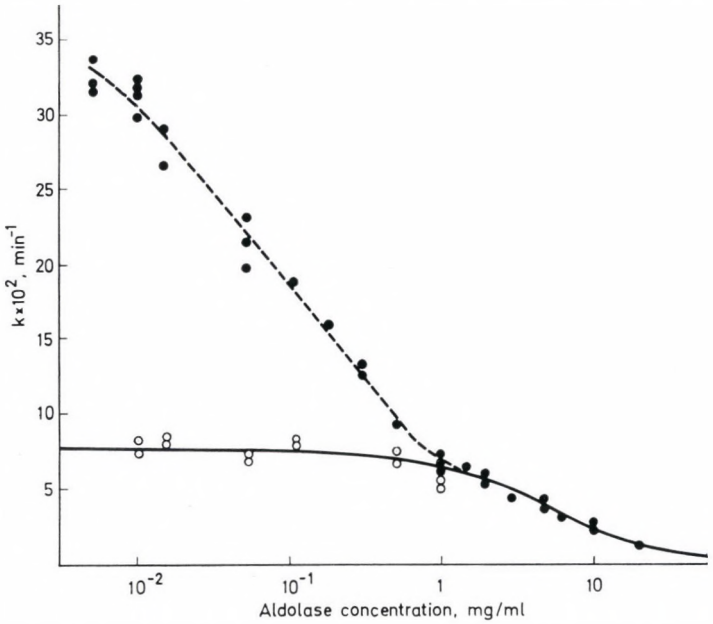

Fig. 3. Dependence of the first order rate constant of inactivation on aldolase concentrations ●—●: irradiation in 0.2 M Tris · HCl buffer, pH 7.5; ○—○: irradiation in the same buffer containing 1% serum albumin. The solid line is the theoretical curve of equation (3)

Three out of the four monomers thus produced are fairly intact and at high aldolase concentration rapidly recombine forming new tetramers. However, towards low enzyme concentrations the extent of recombination gradually decreases, because the unstable monomers are denatured before they could recombine. If serum albumin is present, it prevents this secondary denaturation and the inactivation rate constant increases only slightly as predicted by the theoretical curve.

3. Hybridization of native and succinyl aldolase by UV irradiation

Mechanism *b)* could be tested experimentally, since if aldolase subunits dissociate and recombine upon irradiation, the enzyme should hybridize with a suitable isoenzyme partner provided that the latter also undergoes dissociation-

recombination under such conditions. We used a chemically modified form of aldolase, succinyl aldolase (Meighen, Schachman, 1970) as hybridizing partner. It was found that the two species formed hybrids if their mixture was irradiated with ultraviolet light. As shown in Fig. 4, beside the homotetramer peaks, three somewhat blurred but recognizable peaks corresponding to the three heterotetramers appeared in the gel-electropherogram. The hybrid peaks were absent if the mixture was incubated without irradiation.

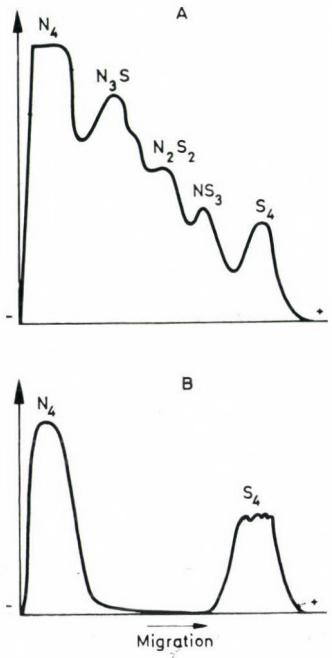

Fig. 4. Densitometric tracing of the gel-electrophoretic pattern of aldolase and succinyl aldolase mixtures. Aldolase, 1 mg/ml; succinyl aldolase, 0.5 mg/ml. Reanal Modell 69 disc-electrophoretic apparatus. A: Mixture irradiated with UV light for 6 min; B: Mixture incubated for 24 hours at 25°C without irradiation

In conclusion, the experiments are consistent with our earlier result (Friedrich et al., 1972), according to which aldolase does not undergo spontaneous dissociation down to about 5 μg/ml protein concentration. However, the enzyme can be induced by UV light to dissociate and a part of the monomers thus produced are capable of recombination.

Thanks are due to Prof. F. B. Straub and Dr Gertrud Szabolcsi for continual interest and helpful discussions.

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Comparison of Trypsin Activated by Alkylammonium Ions and α-Chymotrypsin

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Steady state kinetic measurements were carried out on the hydrolyses of N-acetylglycine, N-acetyl-L-alanine and N-acetyl-L-valine ethyl esters by trypsin, trypsin and alkylammonium ions and α-chymotrypsin at pH 6.6 and 25°C. The following alkylamines were tested: methyl-, ethyl- and propylamines. These effectors cause activation or inhibition of the catalysis depending on the substrate and amine.

For the interpretation of the results it has been assumed that trypsin can exist in a less and more active (α-chymotrypsin-like) form. The interconversion of the less active to the more active conformation seems to be brought about by the positive charge of the side chains of lysine and arginine substrates or that of alkylamines.

Introduction

The different substrate specificities of α-chymotrypsin and trypsin are due to the change of one amino acid of their sequences, namely, the Ser-189 of α-chymotrypsin has been replaced by Asp-189* in trypsin. The substrate-binding pocket of α-chymotrypsin was shown by electron density map and skeletal model (Steitz et al., 1969; Birktoft et al., 1970). Recently the crystal structure of DIP-trypsin has been investigated at 2.7 Å resolution (Stroud et al., 1969; 1971). The substrate-binding pocket of trypsin contains the negatively charged carboxylate group of Asp-189 located at the bottom and a chymotrypsin-like hydrophobic region. The catalytic site is at the entrance of the pocket. The side chains of the specific, positively charged trypsin substrates (lysine or arginine) are bound in an extended conformation forming a salt linkage with the carboxylate group and apolar interactions with the hydrophobic lining of the pocket. These binding factors influence the orientation of the substrate, i.e. the efficiency of the catalytic machinery of the enzyme on the peptide, amide or ester bond of the basic amino acid.

* Residues are numbered according to the sequence of bovine chymotrypsinogen A (Hartley, 1970). Abbreviations not listed in Biochemistry *5* 1445 (1966): AAEE, N-acetyl-L-alanine ethyl ester; AGEE, N-acetylglycine ethyl ester; ATEE, N-acetyl-L-tyrosine ethyl ester; AVEE, N-acetyl-L-valine ethyl ester; BAEE, Nα-benzoyl-L-arginine ethyl ester; CR, α-chymotrypsin; ETA, ethylammonium; META, methylammonium and PROPA, 1-propylammonium chloride; TPCK, L-(1-tosylamido-2-phenyl)-ethyl chloromethyl ketone; TR, trypsin.

Inagami and Murachi (1964a), Inagami and Hatano (1969) proposed that the binding of a positively charged side chain to trypsin induces a conformational change of the enzyme resulting in the increased activity of its catalytic site. One can study this effect of the NH_3^+ group on the activity of trypsin by taking a specific substrate like N-acetyl-L-lysine ethyl ester into two parts, i.e. into a neutral amino acid and an alkylamine part. Inagami and Murachi (1964a), then Erlanger and Castleman (1964) and recently Seydoux (1969), Seydoux et al. (1971) showed that alkylammonium ions increased the rate of the trypsin-catalyzed hydrolysis of acetylglycine esters. To gain some further information about the mechanism of the specificity of trypsin it seems interesting to split the side chain of lysine not only at the $\alpha-\beta$ carbon bond, but also at the $\beta-\gamma$ bond and so forth, i.e. to use other neutral substrates instead of glycine.

In the present work a kinetic study was made on the effect of methyl, ethyl and propylammonium ions on the hydrolysis of N-acetylglycine, -L-alanine and -L-valine ethyl esters catalyzed by trypsin. The steady-state parameters of trypsin obtained in the presence of alkylammonium ions were compared with those of trypsin and α-chymotrypsin in the absence of effectors. Several data already published in a preliminary communication (Vajda, Szabó, 1971) have been reinvestigated, recalculated and completed. The results show that the activity of trypsin towards neutral substrates is increased by some alkylammonium ions.

Experimental

Materials

Substrates – Melting points were measured in a Tottoli type apparatus (Büchi, Switzerland) and are uncorrected. Thin- layer chromatography (tlc) was performed on silica gel G (Reanal, Hungary) plates (2.5 × 7.5 cm) in the following solvent systems: a, 2-n-butanol – 3% NH_4OH, 100 : 44; b, 2-n-butanol-2-propanol-chloroacetic acid – water, 70 : 10 : 3 g : 40; c, acetonitrile – acetic acid – chloroform, 60 : 10 : 30. Spots were detected with ninhydrin and chlorine-tolidine.

The substrates were prepared on the 0.2 – 0.4 mole scale.

N-acetylglycine ethyl ester was synthesized according to the method of Inagami and Mitsuda (1964b). It was distilled twice, b.p. 130 – 134°C/1.9 mm (reported 121°C/1.3 mm) and then melted at 48 – 49°C (reported 47.5 – 48°C). Tlc a, R_f 0.7; b, 0.7; c, 0.7 a single ninhydrin-negative, chlorine-positive spot.

Analysis calculated for $C_6H_{11}NO_3$: C, 49.46; H 7.64; N 9.65; C_2H_5O 31.04. Found: C, 49.30; H, 8.54; N, 9.71; C_2H_5O 30.89 and for another batch: C, 49.99; H, 7.62; N, 9.48; C_2H_5O 31.12.

In some cases the distilled material contained traces of acidic impurity and therefore its concentrated aqueous solution was neutralized to pH 6.5 with 1 N NaOH and then extracted into dichloro-methane. The organic layer was evaporated in vacuo and dried over P_2O_5 in a desiccator. The same procedure was used also for the other substrates.

N-acetyl-L-alanine-ethyl ester was prepared by esterification and then acetylation of L-alanine. The esterification was carried out in the ordinary way (Greenstein, Winitz, 1961) by bubbling hydrogen chloride gas into the ethanolic suspension of the amino acid. The solution obtained was concentrated in vacuo and then dissolved in ethanol and evaporated again. The crude ester hydrochloride in dichloro-methane (0.5 M) was kept at 0 – 5°C and stirred with 1.5 equivalents of triethylamine. To this mixture 1.5 – 1.5 equivalents of acetyl chloride and triethylamine were added dropwise successively. The reaction mixture was further stirred for 1 hr with cooling and finally for 3 hrs at room temperature. Then it was left at room temperature for another day. The crude mixture was filtered with suction and the filtered solution was washed with water, dilute HCl solution, saturated $NaHCO_3$ solution and water. The volumes of the washing solutions were around 10% of the dichloro-methane solution. The dried organic part was evaporated in vacuo and the remaining crude product distilled, b.p. 76 – 80°C/0.05 – 0.10 mm (reported: 80°C/0.1 mm by Winterstein et al., 1956). The yield after two recrystallizations from ether-petroleum ether was 80% as calculated for the starting amino acid. The product melted at 32 – 33°C (reported: 34 – 35°C by Cohen et al., 1961); $[\alpha]_D$ −81.3°, c 3 in H_2O (reported: −80°, c 3 in H_2O by Winterstein et al., 1956). Tlc a, R_f 0.8; b, 0.7; c, 0.8 a single ninhydrin-negative, chlorine-positive spot.

Analysis calculated for $C_7H_{13}NO_3$: C, 52.82; H, 8.23; N, 8.80. Found: C, 53.10; H, 8.50; N, 8.95.

N-acetyl-L-valine ethyl ester was synthesized as described above for N-acetyl-L-alanine ethyl ester. The difference between the two procedures was only that the ester-hydrochloride remained as a suspension in ethanol. It was distilled at 88 – 91°C/0.05 – 0.10 mm (reported: 100 – 102°C/0.75 mm by Bergel et al., 1962) to yield 62%, m.p. 32.5 – 33.5°C (reported: 32.5 – 34.2°C by Waite and Niemann, 1962), $[\alpha]_D$ −48.6°, c 5.2 in H_2O (reported: −50.3°, c 4.5 in H_2O by Waite and Niemann, 1962). Tlc a, R_f 0.7; b, 0.7; c, 0.7 a single ninhydrin-negative, chlorine-positive spot.

Analysis calculated for $C_9H_{17}NO_3$: C, 57.73; H, 9.15; N, 7.48. Found C, 57.60; H, 9.87; N, 7.13 and for another batch: C, 57.49; H, 9.43; N, 7.61.

BAEE was purchased from Koch-Light Laboratories and ATEE from Calbiochem.

The stock solutions of substrates in water were the following. AGEE: 5.0 M, AAEE: 2.5 M, AVEE: 1.3 and 2.5 M. If kept over 2 – 3 weeks in the refrigerator the solutions became a bit acidic (e.g. the acid content of a 0.5 M solution of AVEE changed from 0.2% to 0.8% in 50 days). *Effectors* – Methylamine (purum) was an aqueous solution of about 40%, obtained from Fluka. The stock solution of methylammonium chloride was prepared by diluting the aqueous solution with water to a given volume, then its concentration was determined by titration of an aliquot and finally the main part neutralized and diluted to pH 6.2 and 2.0 M. The ethylammonium chloride solution was obtained either from ethylamine as an anhydrous liquid under pressure (Fluka,

purum) or from crystalline hydrochloride (Fluka, puriss.). The aqueous solution of the amine was neutralized and diluted to pH 6.6 and 0.5 M as above, whereas the crystalline sample was used without further neutralization as a 1.0, 2.0 or 4.0 M solution. From 1-propylamine, which was an anhydrous liquid (Fluka, purum) a pH 6.5 and 0.1 M hydrochloride stock solution was prepared.

The stock solutions of amines could be stored indefinitely in the refrigerator.

Enzymes – Trypsin was a twice re-crystallized, salt-free, lyophilized Koch-Light preparation (Lot No. 48456). Concentrations of the trypsin solutions were determined on the basis of optical density at 280 nm by using $E^{280}_{1\%} = 14.4$ (Davie, Neurath, 1955) and assuming a molecular weight of 23 000 based on amino acid analysis (Dayhoff, Eck, 1968). The tryptic activities of the trypsin prepara-

Table 1

Comparison of initial rates of hydrolyses catalyzed by commercial and TPCK-treated trypsin

The rate of hydrolysis of 0.001 M ATEE as a function of enzyme concentration was determined by the decrease in absorbance at 237 nm in a 1 cm cuvette in 0.05 M phosphate buffer, pH 7.0, at room temperature; volume 3 ml; readings were taken every minute over a 7-minute period with a Beckman DU spectrophotometer. The initial rates of hydrolysis are expressed as change in absorbance per minute.

Enzyme	$E^{237}_{1\,cm}$/minute	
	Enzyme concentrations (mg/3 ml)	
	0.17	0.25
Commercial TR	0.007	0.010
TPCK-treated TR	0.006	0.008

tions were measured with 0.009 M BAEE at 25°C and pH 8.0 in 0.1 M KCl in a pH-stat (0.1 N NaOH). The specific activities were around 30 (μeq/min/mg enzyme). Trypsin 0.02 g (the same lot as above) was treated with TPCK as described by Kostka and Carpenter (1964) and the stock solution obtained was used on the day of treatment without dialysis and lyophilization. For comparison another 0.02 g of trypsin was treated in the same way without TPCK ("Commercial TR" in Table 1). The chymotryptic activities of trypsin and TPCK-trypsin were determined with 0.001 M ATEE, pH 7.0, spectrophotometrically (Table 1), according to Kostka and Carpenter (1964).

α-Chymotrypsin was a three times recrystallized, salt-free, lyophilized Koch-Light preparation (Lot No. 93141). $E^{280}_{1\%} = 20.0$ (Dixon, Neurath, 1957) and a molecular weight of 25 000 (Wilcox et al., 1957) were used for calculations. Activity measurements were performed at a concentration of 0.018 M of ATEE in 5% (v/v) dioxane at 25°C and pH 8.0 in 0.1 M KCl by pH-stat titration (0.1 N NaOH). The specific activities were around 370 (μeq/min/mg enzyme).

Distilled and deionized (Elga) water was used.

Methods

Kinetic procedure – Initial rates of hydrolysis were determined by the pH-stat method with a Radiometer automatic titrator (model TTT1c) and recorder (model SBR2c) with a reaction volume of 4 ml in a 15 ml reaction vessel and 0.02 N NaOH as titrating agent. The alkali was delivered by a Radiometer syringe burette and restandardized daily with hydrochloric acid. In the jacket of the reaction vessel water was circulated at 25.0 ± 0.1°C. Inserted into the vessel were a glass electrode (Radiometer G 202 B) and a calomel reference electrode, a stirrer and through a common opening the tubes for the titrant and nitrogen. The same slot was used for the injection of the enzyme solution. The nitrogen had been bubbled through water before passed in a slow stream over the surface of the reaction mixture. All experiments were carried out at pH 6.6 ± 0.3. At this pH the nonenzymatic hydrolysis of substrates catalyzed by hydroxide ions was negligible as shown by Inagami and Murachi (1964a) for AGEE and confirmed also by us for AGEE, AAEE and AVEE. Inagami and Murachi (1964a) also excluded the amidation by alkylamines.

The concentration of the stock solutions of trypsin and α-chymotrypsin was around 3×10^{-3} M in 0.001 N HCl. The stock solutions were prepared daily. Enzyme concentrations of the reaction mixture were around 4×10^{-5} M. The enzyme solution was added to the reaction mixture with a 50 μl micropipet. The solutions were 0.1 M in KCl, except where the effect of KCl on the rate of hydrolysis was studied (see Fig. 1).

The pH of the enzyme solution was not previously adjusted to 6.6 but the solution was titrated in the pH-stat, which gave a flat curve on the titrigraph and at the end of this titration the slope of the curve suddenly became much steeper showing the hydrolysis of substrate. Thus it was easy to recognize which portion of the curve should be considered as the trace of the substrate hydrolysis. If substrate hydrolysis proceeded slowly, first the 0.5 proportional band was used and only after getting to pH 6.6 (end point of pH adjustment) was the apparatus switched to a higher sensitivity. On the other hand, if the reaction rate was fast, the enzyme and substrate were titrated at the proportional band which was suitable to follow the hydrolysis of substrate. The volume of sodium hydroxide consumed by the titration of enzyme was taken into account. The self-digestion of enzymes at pH 6.6 ("enzyme blank") during the 4–8 minutes period of observation was practically negligible.

In a typical experiment, substrate and effector solutions, 0.4 ml of 1.0 M KCl solutions and water were mixed in the cell and the pH was adjusted to 6.6 by the automatic titrator. The reaction was then started by the addition of 50 μl of the enzyme solution and followed up to 4–8 minutes. The total volume of the reaction mixture at the start was 4 ml (including also the amount of NaOH solution used for the titration of enzyme).

Calculations – A general mechanism of trypsin or α-chymotrypsin-catalyzed hydrolysis can be described by the classical three-step scheme (Gutfreund, Sturtevant, 1956; Niemann, 1964; Bender, Kézdy, 1965):

$$\overset{k_{cat}}{\longrightarrow}$$

$$E + S \overset{K_s}{\rightleftharpoons} ES \overset{k_2}{\longrightarrow} ES' \overset{k_3}{\longrightarrow} E + P_2 \qquad (1)$$

$$+ P_1$$

$$\overset{K_{m,\,app}}{\longrightarrow}$$

where E and S are the enzyme and substrate concentrations, ES is the Michaelis complex and ES' is the acylenzyme. P_1 and P_2 are products (alcohol and acid). The substrate is in a large excess to the enzyme. The measurable steady-state parameters k_{cat} and $K_{m,\,app}$ are related to the k_2 and k_3 rate constants and to the K_s dissociation constant in the following way:

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \qquad (2)$$

$$K_{m,\,app} = \frac{K_s k_3}{k_2 + k_3}. \qquad (3)$$

A more complex four-step mechanism has also been proposed as shown partly in eq.(4) (Gutfreund, 1971):

$$\overset{k_2}{\longrightarrow}$$

$$ES \overset{k_{2a}}{\rightleftharpoons} ES^* \overset{k_{2b}}{\longrightarrow} ES' \qquad (4)$$

$$+ P_1$$

where ES^* refers to an activated complex and so the k_2 acylation rate constant is divided into k_{2a} isomerization and k_{2b} acylation parts.

A minimal general reaction scheme including an EAS ternary complex formation from trypsin, A (alkylammonium ion) and substrate is given by the combination of eqs (1) and (5).

$$E \overset{K_s}{\rightleftharpoons} ES \overset{k_2}{\longrightarrow} ES' \overset{k_3}{\longrightarrow} E + P_2 \qquad (1)$$

$$K_A \uparrow\downarrow$$

$$EA \underset{K_s'}{\rightleftharpoons} EAS \overset{k_2'}{\longrightarrow} EAS' \overset{k_3'}{\longrightarrow} EA + P_2. \qquad (5)$$

The binding of A to ES seems unlikely.

The combinations of the two equilibria means that the reaction mixture contains both ES and EAS complexes.

The steady-state treatment of the mechanism shown in eq.1 leads to the Michaelis–Menten equation or to its linearized form, i.e. to the Lineweaver–Burk plot as given in eq. (6):

$$\frac{1}{v_0} = \frac{K_{m,\,\mathrm{app}}}{k_{cat}[E_0][S]} + \frac{1}{k_{cat}[E_0]} \tag{6}$$

where v_0 refers to the initial rate measured as P_2 and E_0 is the total enzyme concentration.

On the other hand, if we consider: the K_s, K'_s and K_A dissociation constants of eqs (1) and (5) and the velocity equation

$$v_0 = k_3[ES'] + k'_3[EAS']$$

and that

$$[E_0] = [E] + [ES] + [ES'] + [EA] + [EAS] + [EAS']$$

then the solution of the corresponding equations gives, after substituting $K_{m,\,\mathrm{app}}$ and $K'_{m,\,\mathrm{app}}$ for K_s and K'_s and k_{cat} and k'_{cat} for k_3 and k'_3 respectively, according to eqs (2) and (3):

$$\frac{1}{v_0} = \frac{[S][A]K_{m,\,\mathrm{app}} + [S]K'_{m,\,\mathrm{app}}K_A + [A]K_{m,\,\mathrm{app}}K'_{m,\,\mathrm{app}} + K_{m,\,\mathrm{app}}K'_{m,\,\mathrm{app}}K_A}{[S][E_0]([A]k'_{cat}K_{m,\mathrm{app}} + k_{cat}K'_{m,\mathrm{app}}K_A)} \tag{7}$$

In the present work two types of series were used (Table 2): (i) a range of substrate concentration without alkylamine; (ii) a range of substrate concentration at one fixed alkylamine concentration and a range of alkylamine concentration at one fixed substrate concentration. The first type served to compute the k_{cat} and $K_{m,\,\mathrm{app}}$ parameters according to eq. (6) and the second to get the k'_{cat}, $K'_{m\ \mathrm{app}}$ and K_A constants from eq.(7) (Tables 3–4). To solve eq.(7) the k_{cat} and $K_{m,\,\mathrm{app}}$ values were taken from eq.(6). In our preliminary communication also measurements in the presence of alkylammonium ions were calculated with the help of eq.(6) because of the limited number of data. Therefore those values represent only an average of the ES and EAS reaction pathways.

In the present study all experimental data were analyzed by the iterative, non-linear least square method (maximal number of iterations: 50) on a Razdan-3 computer by using an Algol-type program written by P. Mezey in this Institute (to be published). The program fits experimental data either to eq.(6) or eq.(7).

Results

Effects of methylammonium ion and KCl on the rate of hydrolysis

The effect of methylammonium ion on the relative initial rate of trypsin-catalyzed hydrolysis of AAEE and AVEE, was compared with the effect of KCl (above 0.1 M). Substrate concentrations were taken constant in every case.

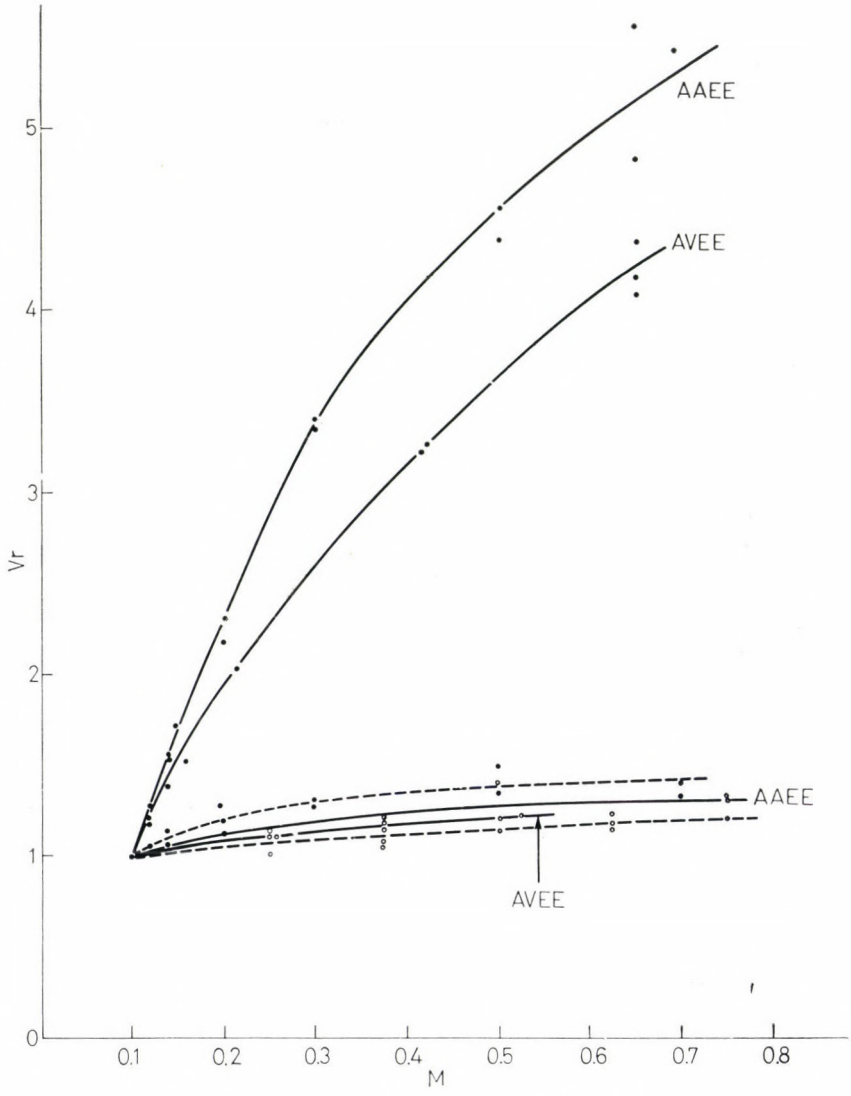

Fig. 1. Effect of methylammonium ion (full circles) and KCl (open circles) on the relative initial rate of the hydrolysis of AAEE catalyzed by trypsin, ——, and chymotrypsin, – – –, and of AVEE catalyzed by trypsin, in 0.1 M KCl at pH 6.6 and 25°C. Substrate concentrations: 0.1 M AAEE and 0.2 M AVEE for methylammonium ion and 0.2 M AAEE and 0.4 M AVEE for KCl. The initial rate in the absence of alkylammonium ion or KCl (above 0.1 M), is taken as unity

Table 2

Range of substrate and effector concentrations

Series			Run	
N-acetyl-amino acid ethyl ester	Enzyme	Amine	N-acetylamino acid ethyl ester concentration (M)	Amine concentration (M)
Gly	TR		0.10—3.20	—
	TR	META	0.10—3.20 1.00	0.40 0.02—0.80
	TR	ETA	0.10—3.20 1.00	0.10 0.005—0.24
	TR	PROPA	0.10—3.20 1.00	0.02 0.001—0.05
	CR		0.05—2.60	—
Ala	TR		0.05—2.00	—
	TR	META	0.05—1.40 0.50	0.40 0.02—1.20
	TR	ETA	0.05—1.75 0.75	0.20 0.01—0.25
	TR	PROPA	0.13—2.00 0.75	0.03 0.001—0.056
	CR		0.05—1.50	—
Val	TR		0.05—1.50	—
	TR	META	0.013—0.73 0.25	0.60 0.02—0.80
	TR	ETA	0.05—0.83 0.38	0.10 0.005—0.28
	CR		0.013—0.75	—

The relative initial rate, v_r, refers to the initial rate in 0.1 M KCl. The abscissa of Fig. 1 represents the sum of the concentrations of 0.1 M KCl and methylammonium ion or that of 0.1 M KCl and excess KCl. Plots of Fig. 1 illustrate well that whereas methylammonium ion (full circles) at 0.1 M AAEE or 0.2 M AVEE increases the rate 4—5-fold, the effect of KCl (open circles) remains under 1.4-fold at 0.2 M

AAEE or 0.4 M AVEE. These effects have been checked also for α-chymotrypsin. The dashed plots of Fig. 1 show that neither methylammonium ion at 0.1 M AAEE, nor KCl at 0.2 M AAEE has any important effect on the relative initial rate of hydrolysis catalyzed by α-chymotrypsin.

Comparison of commercial and TPCK-treated trypsin. – All the data shown in Table 1 are mean values of three determinations within an error of ± 10%. This means that the possible chymotryptic contamination of the commercial trypsin can be in the order of the error.

Effects of alkylammonium ions on kinetic parameters. – The concentrations of substrates and effectors were as given in Table 2. One run consisted of twenty points measured in parallel experiments. The standard error from the mean values of the parallel experiments was ±9%. The standard deviation of the v_0 values computed from eqs (6) and (7) by using the parameters of Table 3 is ±7% from the observed experimental points. This standard error represents the average of all series.

As shown in Table 3 both the rate and the apparent Michaelis constants are changed by alkylammonium ions. Inagami and Murachi (1964) in the case of AGEE found only the rate constant to change.

Table 3

Kinetic parameters for the hydrolysis of N-acetylamino acid ethyl esters catalyzed by trypsin, trypsin and alkylammonium ions and chymotrypsin

The initial rates of hydrolysis were determined by pH-stat titration at pH 6.6 and 25°C in 0.1 M KCl; $E_0 \sim 4 \times 10^{-5}$ M; volume 4 ml; titrant: 0.02 N NaOH

N-acetyl-amino acid ethyl ester	Enzyme	Amine	k'_{cat} (sec^{-1})	$K'_{m,\,app}$ (M)	$k'_{cat}/K'_{m,\,app}$ ($M^{-1}sec^{-1}$)
Gly	TR*		0.007	1.02	0.007
	TR	META	0.013	0.66	0.019
	TR	ETA	0.070	0.96	0.073
	TR	PROPA	0.018	0.87	0.020
	CR*		0.016	0.50	0.032
Ala	TR*		0.42	0.54	0.78
	TR	META	1.68	0.22	7.70
	TR	ETA	0.81	0.44	1.84
	TR	PROPA	0.13	1.34	0.10
	CR*		0.34	0.52	0.65
Val	TR*		0.02	0.21	0.10
	TR	META	0.10	0.09	1.11
	TR	ETA	0.01	0.30	0.03
	CR*		0.05	0.10	0.50

* Data refer to k_{cat} and $K_{m,\,app}$ parameters (see eq. 1).

As already mentioned above whereas the data of our preliminary communication reflect only the average of the hydrolyses through *ES* and *EAS* complexes, eq.(7) of the present paper made it possible to distinguish the reaction pathway of *EAS* from that of the mixture of *ES* and *EAS*. Therefore some conclusions stated before should be revised, but in spite of this both studies suggest a similar interpretation of the specificity indexes, $k_{cat}/K_{m,\,app}$ and $k'_{cat}/K'_{m,\,app}$. Namely, alkylammonium ions as activators increase the specificity indexes of trypsin.

The binding of alkylammonium ion to free trypsin was also computed and characterized by the K_A dissociation constant. These data, together with some data of the literature, are given in Table 4. The about one order decrease in K_A brought about by one additional CH_2 group of the alkyl chain is in good agreement with a 1.4 kcal/mole binding energy (Baker, 1967).

Table 4

Dissociation constants of trypsin-alkylammonium ion complexes

K_A was determined from the acceleration or inhibition of the initial rates of hydrolysis of N-acetylamino acid ethyl esters.

N-acetylamino acid ethyl ester	Amine	Acceleration	Inhibition
Gly*	META	0.29	
	ETA	0.07	
	PROPA	0.010	
Ala	META	0.17	
	ETA	0.05	
	PROPA		0.005
Val	META	0.27	
	ETA		0.03

* Inagami and Murachi's (1964a) reported values for META, ETA and PROPA were: 0.38, 0.07 and 0.009, respectively.

Discussion

Inagami and Murachi (1964a) have shown that whereas the methyl-, ethyl- and propylammonium ions are activators of the trypsin-catalyzed hydrolysis of N-acetyl-glycine ethyl ester, butylamine has an inhibitory effect. Inagami contrived from these observations a model, according to which the alkyl chains of the alkylammonium ion and of the substrate be side by side in the binding site of trypsin. The inhibition by butylammonium ion should be the result of a slight overlap of the hydrogen of the glycine CH_2 and the terminal CH_3 of butyl. Our results satisfy this model, i.e. the longer is the side chain of the substrate, the

shorter alkyl chain of the alkylamine inhibits.* These observations might reflect an additivity namely that if the lengths of the alkyl chains of the neutral substrate and alkylammonium ion equal that of lysine, inhibition occurs whereas shorter chains cause the activation of trypsin. This interpretation involves that the primary binding site of a neutral substrate is the same as that of the apolar part of a specific one.

The possibility of a secondary binding site outside the active center cannot be excluded. A different model has also been suggested, where the neutral substrates are bound to a site of the enzyme other than the specific one (Sanborn, Hein, 1968; Erlanger, Castleman, 1964; Seydoux, 1969; Seydoux et al., 1971).

The present results showed that (i) the specificity index of neutral substrates is always smaller for trypsin than for α-chymotrypsin and (ii) the alkylammonium ions (of a given size of chain) increase this index from trypsin toward α-chymotrypsin.

Bender et al. (1964) have shown with the p-nitrophenyl esters of N-acyl-leucine, -tyrosine and -tryptophan substrates that the corresponding k_{cat} values are nearly the same for the trypsin and α-chymotrypsin catalyzed hydrolyses. For p-nitrophenyl esters $k_2 \gg k_3$ therefore $k_{cat} = k_3$ (eq.2). This suggests that the differences between the k_{cat} values of ethyl esters hydrolyzed in the presence of trypsin and α-chymotrypsin are realized in the k_2 step. In this step more exactly in the k_{2a} isomerization step (eq.4), the NH_3^+ group of the effector or of the specific substrate might produce its effect on trypsin. This hypothesis cannot be tested by steady-state techniques and should be investigated by rapid reaction methods (possibly with substrates possessing spectrally detectable groups; Bernhard, Gutfreund, 1970).

On the exterior of the substrate-binding pocket of the active form of chymotrypsin the $-COO^-$ of Asp-194 and the $-NH_3^+$ group of Ile-16 form an ion-pair, which has an important effect on the catalytically active conformation of the enzyme (Hess et al., 1970; Birktoft et al., 1970; Fersht, Requena, 1971)**. In the case of trypsin the bottom of the pocket contains the $-COO^-$ group of Asp-189, which means a free charge in a medium of low dielectric constant. It seems likely that this negative charge of the free enzyme is compensated internally by some buried water molecules and also at the expense of some slight conformational rearrangement. Stroud et al. (1971) found in crystallographic studies of DIP-trypsin that the $-COO^-$ group of Asp-189 is hydrogen-bonded to Gln-221A and that the distance between the carboxyl group of Asp-189 and the α-amino of Ile-16 is just 6.5 Å.*** This means that the $-COO^-$ of Asp-189 may be close

* It seems more suitable to use α-amino-butyric acid as substrate instead of valine. Such experiments are in progress and the preliminary results are similar to the data of this paper.

** It should be mentioned that Blair et al. (1971) deny the importance of the positively charged amino group of Ile-16 and support their statement by the retention of activity of α-chymotrypsin after succinylation of the amino group of Ile-16.

*** Dr R. M. Stroud's information.

enough to the Asp-194-Ile-16 ion pair to cause some electrostatic interaction between these three charged groups (Kasserra, Laidler, 1969). No doubt, the formation of an Asp-194–Ile-16–Asp-189 "salt bridge" may result in a slight distortion of the active center of the free trypsin as compared with that of chymotrypsin. The cationic part of the specific substrates or alkylammonium ions interacting with the $-COO^-$ group of Asp-189 seems to abolish the disturbing effect of this Asp-189 and so result in a chymotrypsin-like arrangement of the Asp-194–Ile-16 salt-pair. Perhaps this subtle change involves also the disruption of the Asp-189–Gln-221 A hydrogen bond mentioned above. The slight conformation change adjusts the important groups in the catalytic and binding sites in a manner so that they become similar to those of chymotrypsin, and in this way the catalytic activity is switched on.

This interpretation implies that trypsin exists in two interconvertible conformations, i.e. in a less active and in a more active, chymotrypsin-like one.* The conversion to the latter form seems to be effected by the properly located cationic group of the substrate.

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* The inactive conformation of chymotrypsin existing to some extent also at neutrality (Fersht, Requena, 1971) has not been considered in our interpretation.

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Effect of Conformational Changes on the Enzymatic Inactivation of Pig and Rabbit Skeletal Muscle Phosphorylase-*a*

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We studied the effect of glucose-6-phosphate, glucose, caffeine and AMP on the phosphorylase-phosphatase reaction with pig and rabbit skeletal muscle phosphorylase-*a* as substrates. The results obtained with rabbit muscle phosphorylase-*a* confirm previous observations (Bot, Dósa 1971). Similar results were obtained with pig muscle phosphorylase-*a* except the effect of glucose-6-phosphate. Glucose and caffeine increase the rate of dephosphorylation, whereas AMP decreases it. Glucose-6-phosphate does not alter the rate of dephosphorylation of pig muscle phosphorylase-*a* and does not protect it from inhibition by AMP.

The limited tryptic digestion of pig muscle phosphorylase-*a* in the presence of the above effectors was also studied. AMP had a protective effect, whereas glucose and caffeine enhanced the proteolytic process just as with rabbit muscle phosphorylase-*a*. In contrast to these similarities glucose-6-phosphate did not influence the rate of digestion of pig muscle phosphorylase-*a* and the protective effect of AMP could be partially suspended only by glucose and caffeine and not by glucose-6-phosphate.

The parallel behaviour of the two kinds of phosphorylases in the presence of AMP, glucose and caffeine, and the different behaviour of the two phosphorylases in the presence of glucose-6-phosphate confirmed the hypothesis that the phosphorylase-phosphatase reaction was influenced by the conformational state of phosphorylase-*a* used as substrate.

Introduction

It is known that phosphorylase-phosphatase (EC, 31.3.17) converts the active phosphorylase-*a* into inactive phosphorylase-*b* by removing the inorganic phosphate from the phosphorylated enzyme.

Numerous authors have studied this dephosphorylating reaction, applying rabbit skeletal muscle phosphorylase-*a* as substrate, and have established that several effectors influence the phosphatase reaction. AMP in a concentration of 10^{-5} M inhibits the dephosphorylating reaction (Sutherland, Cori, 1951; Hurd et al., 1966). According to Nolan et al. (1964) AMP acts on phosphorylase-*a* modifying thereby the rate of phosphorylase-phosphatase reaction. Bot, Dósa (1967, 1971) have thoroughly investigated the inhibitory effect of AMP on phosphatase reaction and verified the assumption of Nolan et al. (1964).

Although several authors have observed that one or another intermediate of glycogenolysis affects the phosphorylase-phosphatase reaction, very little is

known about the mechanism of action of these effectors. The influences of some intermediates have been summarized by Bot and Dósa (1967, 1971) and Bot (1970).

The NaF inhibition of phosphorylase-phosphatase reaction was first reported by Sutherland and Cori (1951) and Keller and Cori (1955). Bot and Dósa (1967, 1971) have shown that NaF inhibits the action of phosphatase. Recently Goris et al. (1972) reported that NaF reversibly inactivates the phosphatase.

In the present work the conversions of pig and rabbit muscle phosphorylase-*a*'s have been compared in the presence of glucose-6-phosphate, glucose, caffeine and AMP. The behaviour of pig phosphorylase-*a* as substrate in the phosphatase reaction is noteworthy because the pig enzyme is the only phosphorylase, according to our present knowledge, the activity of which cannot be inhibited by glucose-6-phosphate (Hanabusa et al., 1967). We found earlier (Bot et al., 1973) that the above effectors changed the tryptic susceptibility of rabbit muscle phosphorylase-*a*.

To prove that the effectors influence the rate of dephosphorylation by changing the conformational state of phosphorylase-*a*, we also studied the tryptic digestion of pig muscle phosphorylase-*a*. Tryptic digestibility is a sensitive indicator of slight structural changes of a protein molecule.

Methods

Phosphorylase-b-kinase was prepared from rabbit skeletal muscle according to Krebs et al. (1964). The specific activity of the enzyme was 89 units/mg protein.

Protein concentration was determined by the method of Lowry et al. (1951).

Pig skeletal muscle phosphorylase-b. The procedure described for the isolation of pig skeletal muscle phosphorylase-*b* (Hanabusa et al., 1967) was modified as shown in Table 1.

Rabbit skeletal muscle phosphorylase-a was prepared from crystalline phosphorylase-*b* with activated phosphorylase-kinase according to the method of Fischer and Krebs (1962). The specific activity of the enzyme obtained by this method was 55 units/mg protein.

Pig skeletal muscle phosphorylase-a was obtained from pig muscle phosphorylase-*b* with rabbit muscle kinase by the modified method of Hanabusa et al. (1967). The specific activity of pig muscle phosphorylase-*a* was 45 units/mg protein.

Rabbit and pig muscle phosphorylase-*a*'s obtained by the kinase reaction were crystallized in 0.01 M Tris-0.02 M mercaptoethanol–0.002 M EDTA buffer, pH 6.8, then treated with Norit to remove traces of AMP.

Protein concentration was determined on the basis of absorbancy at 280 nm using the absorbancy coefficient $A_{280}^{1\%} = 11.7$ (Velick, Wicks, 1951).

Preparation of phosphorylase-phosphatase

Phosphorylase-phosphatase was prepared by the method of deWulf et al. (1970). A two-fold dilution obtained by gel filtration on a Sephadex G-25 column was used as phosphorylase-phosphatase preparation.

Table 1

Purification of pig skeletal muscle phosphorylase-b

Starting material: 400 g of pig muscle. Units of activity are expressed as micromoles of P_i liberated per minute at 30°C (see Methods)
Protein concentration was determined by the method of Lowry et al. (1951)

Purification	Volume, ml	Phosphorylase activity in the presence of 1 mM AMP, units/ml	Protein, mg/ml	Specific activity, units/mg protein	Total activity, units
Crude extract	1,020	37.1	19.8	1.88	37,842
Supernatant of precipitate (pH 5.1)	1,000	28.2	11.5	2.45	28,200
First $(NH_4)_2SO_4$ precipitate to 0.42 saturation, dialysis	26	379	31.2	12.1	9,854
Heat treatment at 37°C, pH 8.8	26	460	29.9	16.1	11,950*
Heat treatment at 45°C, pH 7.0	26	540	24	22.1	14,040*
Second $(NH_4)_2SO_4$ precipitate to 0.35 saturation, dialysis	20	570	26.2	25.6	13,400

* It is noteworthy that the heat treatment caused an increase in enzyme activity.

Assay of phosphorylase-phosphatase activity

Phosphorylase-phosphatase activity was assayed by incubating phosphorylase-*a* with phosphorylase-phosphatase at 30 °C and measuring residual phosphorylase-*a* activity.

Phosphorylase-*a* solutions of 40 – 50 units/ml were preincubated in 0.01 M Tris–0.002 M EDTA–0.02 M mercaptoethanol buffer, pH 6.8, at 30 °C for 10 minutes. 0.2 ml aliquots were mixed with 0.6 ml phosphorylase-phosphatase dissolved in the above buffer. The concentration of phosphorylase-phosphatase was chosen not to induce more than 50% inactivation of phosphorylase-*a* in 10 minutes. The reaction was stopped by the addition of 0.08 M glycerophosphate – 0.1 M NaF – 0.004 M EDTA, pH 6.8, to attain a dilution in which the activity of the residual phosphorylase-*a* could still be measured.

Assay of phosphorylase-a activity

Phosphorylase-*a* activity was determined in the direction of glycogen synthesis according to the method of Illingworth and Cori (1953). The assay mixture contained 16 mM glucose-1-phosphate, 1% glycogen and an amount of phosphorylase-*a* that converted not more than 20% of glucose-1-phosphate during the incubation period.

The amount of P_i liberated from glucose-1-phosphate was determined according to Taussky – Shorr (1953). One unit of activity is defined as the amount of enzyme liberating 1 μmole of P_i from glucose-1-phosphate per minute.

Tryptic digestion of phosphorylase-a

The limited tryptic digestion of pig skeletal muscle phosphorylase-*a* was studied at 30 °C. The incubation (0.8 ml) mixture contained 20 units of phosphorylase and 10 μg of trypsin in 0.04 M Tris – 0.01 M mercaptoethanol – 0.002 M EDTA buffer, pH 6.8.

Effectors tested during the tryptic digestion were dissolved in the above buffer. At appropriate time intervals samples were taken and diluted with the above buffer containing soy-bean trypsin inhibitor (molar ratio of trypsin/trypsin-inhibitor = 1 : 5). Digestion was followed by measuring the decrease of phosphorylase-*a* activity.

Results

Effect of AMP and glucose-6-phosphate on the dephosphorylation of pig muscle phosphorylase-a

It has been shown that glucose-6-phosphate increases the rate of phosphorylase-phosphatase reaction with rabbit skeletal muscle phosphorylase-*a* as substrate (Bot, Dósa, 1967, 1971). We compared the effect of glucose-6-phosphate on dephosphorylation with pig and rabbit muscle phosphorylase-*a* as substrates (Fig. 1).

Fig. 1 illustrates that glucose-6-phosphate does not affect the rate of the inactivation of pig phosphorylase-*a* (*A*), whereas the inactivation of rabbit muscle phosphorylase-*a* proceeds at a significantly higher rate (*B*).

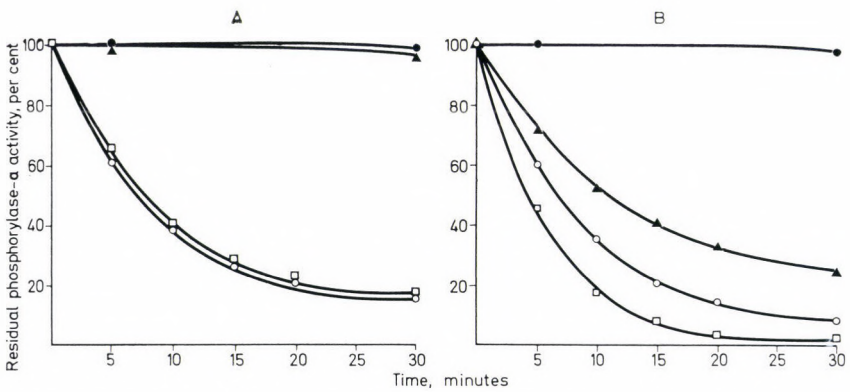

Fig. 1. Effect of glucose-6-phosphate on the inactivation of pig and rabbit skeletal muscle phosphorylase-*a*'-s in the absence and presence of AMP

Pig (*A*) and rabbit (*B*) skeletal muscle phosphorylase-*a*'-s were preincubated in 0.01 M Tris – 0.002 M EDTA – 0.02 M mercaptoethanol buffer, pH 6.8, at 30°C for 10 minutes. 0.2 ml aliquots were mixed with the above buffer and phosphorylase-phosphatase, final volume 0.8 ml. Effectors: none (○ — ○); 20 mM glucose-6-phosphate (□ — □); 10^{-5} M AMP (● — ●); 10^{-5} AMP + 20 mM glucose-6-phosphate (▲ — ▲). Phosphorylase-*a* was diluted and assayed as described in Methods

Pig muscle phosphorylase-*a* is much more insensitive to glucose-6-phosphate in the presence of AMP. It is known that AMP bound to rabbit muscle phosphorylase-*a* protects it from inactivation by phosphatase. The effect of AMP can be moderated by glucose-6-phosphate and high concentrations of glucose-6-phosphate can completely abolish it.

The inactivation of pig and rabbit muscle phosphorylase-*a* in the presence of AMP and AMP + glucose-6-phosphate is also shown in Fig. 1. AMP (10^{-5} M) inhibits the inactivation by phosphatase of both phosphorylases. In the case of pig muscle phosphorylase-*a* the inhibition caused by AMP is not affected by glucose-6-phosphate (*A*), but it is decreased if rabbit muscle phosphorylase-*a* is the substrate (*B*).

Influence of glucose and caffeine on the inactivation of phosphorylase-a

Glucose stimulates the phosphatase reaction with rabbit muscle phosphorylase-*a* and can moderate the inhibition caused by AMP as shown by Bailey and Whelan (1972).

Caffeine enhances the rate of dephosphorylating reaction with liver muscle phosphorylase-*a* substrate (Sutherland, Cori, 1951; Krebs et al., 1964; Stalmans et al., 1970).

Table 2

Effect of glucose and caffeine on the inactivation of pig skeletal muscle phosphorylase-a by phosphorylase-phosphatase

The composition of the reaction mixture and activity assay of phosphorylase-*a* were the same as described in Methods and in Fig. 1

Additions	Pseudo first order rate constants of inactivation $k \times 10^{-3}$ [min^{-1}]	
	Pig muscle phosphorylase	Rabbit muscle phosphorylase
None	72.9	115.3
Glucose (20 mM)	115.5	198.0
Caffeine (5 mM)	138.6	346.5
AMP (10^{-5} M)	1.2	1.2
AMP (10^{-5} M) + glucose (20 mm)	23.1	34.6
AMP (10^{-5} M) + caffeine (5 mM)	34.6	63.0

The different effects of glucose-6-phosphate on the inactivation of pig and rabbit phosphorylase-*a*'s prompted us to study the influence of these effectors on the dephosphorylating reaction. We have also examined the influence of glucose and caffeine on the inactivation of the two phosphorylase-*a*'s in the presence of AMP.

The results summarized in Table 2 show that glucose stimulates the conversion of both muscle phosphorylase-*a*'s to phosphorylase-*b*, and decreases the AMP inhibition. Caffeine, like glucose, increases the rate of the phosphorylase-phosphatase reaction of both phosphorylase-*a*'s and is able to moderate the inhibitory effect of AMP with both phosphorylases.

Influence of AMP and glucose-6-phosphate on the tryptic digestion of muscle phosphorylase

In our previous papers we suggested that the influence of effectors on the dephosphorylation of rabbit muscle phosphorylase-*a* could be due to conformational changes of phosphorylase-*a* used as substrate (Bot, 1970; Bot, Dósa, 1971). To have further evidence for the assumption that the rate of phosphorylase-phosphatase reaction depends on the conformational state of phosphorylase-*a*, we studied the limited tryptic digestion of pig muscle phosphorylase-*a* in the presence and absence of different effectors.

Fig. 2 illustrates that AMP protects the enzyme against the action of trypsin, whereas glucose-6-phosphate has no influence on the tryptic digestion of either native or AMP-treated pig muscle phosphorylase-*a*. In another series of experiments we have shown that glucose-6-phosphate enhances the tryptic digestion of rabbit muscle phosphorylase-*a*, which is sensitive to this effector, and suppresses almost completely the protective effect of AMP (Bot et al., 1973). Thus the two kinds of phosphorylases behaved in a similar manner during tryptic digestion in the presence of AMP whereas in the presence of glucose-6-phosphate this similarity disappeared just as in the phosphorylase-phosphatase reaction.

Effect of glucose and caffeine on the tryptic digestion of phosphorylase-a

We have recently reported that glucose decreases the protective effect of AMP during the digestion of rabbit muscle phosphorylase-*a* (Bot et al., 1973). A similar phenomenon was also observed during the limited tryptic digestion of pig muscle phosphorylase-*a* (Fig. 2).

Caffeine accelerates tryptic digestion not only with rabbit muscle phosphorylase-*a* (Bot et al., 1973), but also with pig phosphorylase-*a*. Caffeine almost entirely abolishes the protection of AMP during the digestion of pig phosphorylase-*a* (Fig. 3).

These results are in good agreement with our data concerning the phosphatase reaction (see Table 2).

Discussion

The results on the tryptic digestion of pig muscle phosphorylase-*a* support the assumption that the effectors influencing phosphatase reaction exert their effects not on the converting enzyme, but on phosphorylase-*a* used as substrate, by inducing conformational changes in the protein.

This conclusion was drawn first of all from the different effects of glucose-6-phosphate on the tryptic digestion of pig and rabbit muscle phosphorylase-*a*'s. In contrast to the effect exerted on the digestion of rabbit muscle phosphorylase-*a* (Bot et al., 1973), glucose-6-phosphate does not influence the rate of digestion of pig muscle phosphorylase-*a* either in the presence or absence of AMP. Accordingly, glucose-6-phosphate is able to cause conformational changes only in rabbit muscle phosphorylase-*a*. That tryptic digestion of pig muscle phosphorylase-*a* is not

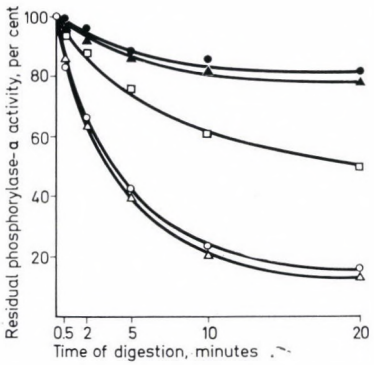

Fig. 2. Limited tryptic digestion of pig skeletal muscle phosphorylase-*a* in the absence and presence of AMP, glucose-6-phosphate and glucose. 20 units of pig muscle phosphorylase-*a* were incubated with 10 μg of trypsin in 0.04 M Tris — 0.01 M mercaptoethanol — 0.002 M EDTA buffer, pH 6.8 at 30°C. Effectors were dissolved in the above buffer, final volume 0.8 ml. No effectors (○ — ○); 10^{-5} M AMP (● — ●); 15 mM glucose-6-phosphate (△ — △); 10^{-5} M AMP + 15 mM glucose-6-phosphate, (▲ — ▲); and 10^{-5} M AMP + 20 mM glucose (□ — □). Phosphorylase-*a* was diluted and assayed as described in Methods

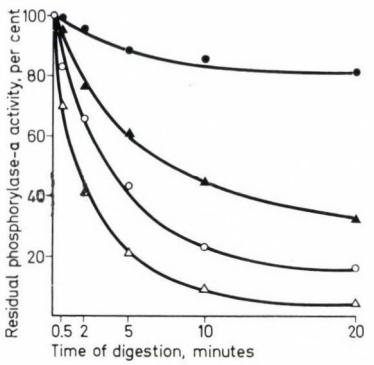

Fig. 3. Effect of caffeine on the limited tryptic digestion of *pig* skeletal muscle phosphorylase-*a* in the presence and absence of AMP. 20 units of pig muscle phosphorylase-*a* were incubated with 10 μg of trypsin. Effectors: none (○ — ○); 10^{-5} M AMP (● — ●); 5 mM caffeine (△ — △); 10^{-5} M AMP + 5 mM caffeine (▲ — ▲)

affected by glucose-6-phosphate can be due to the fact, that glucose-6-phosphate cannot alter the conformational state of pig muscle phosphorylase-*a* either in the native state or in the state induced by AMP.

This also explains the observation that glucose-6-phosphate does not increase the inactivation of pig muscle phosphorylase-*a* by phosphatase either, and because it does not decrease the affinity of pig muscle phosphorylase-*a* to AMP, it does not suppress the inhibitory effect of AMP on the phosphorylase-phosphatase reaction.

The other two effectors, glucose and caffeine, influenced the tryptic digestion of pig and rabbit muscle phosphorylases alike, namely, these effectors caused conformational changes in both phosphorylases. Thus it is conceivable that these effectors influence the dephosphorylation of both phosphorylase-*a*'s and also alleviate the inhibition caused by AMP. The conformational changes of phosphorylase-*a* also manifest themselves in the enzymatic activity in the presence of glucose-6-phosphate, glucose and caffeine. It has been shown that glucose (Helmreich et al., 1967) and caffeine (Kihlman, Overgaard-Hansen, 1955; Alpers et al., 1963) inhibit the activity of rabbit muscle phosphorylase-*a*.

Glucose-6-phosphate only decreases the activity of rabbit muscle phosphorylase-*b* and *a*, while it does not decrease the activity of pig muscle phosphorylase-*a* (Bot et al., 1971; Bot, 1970).

Thus pig muscle phosphorylase-*a* appears to be insensitive to glucose-6-phosphate, which means that glucose-6-phosphate does not induce conformational changes in the protein, consequently it is unable to influence the dephosphorylation reaction.

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Thermoanalytical Investigations on Myosin and the Myofibril

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The structural stability of myosin and myofibrils was studied by the thermoanalytical method. Myosin and its chromatographic fractions showed definite decomposition rate maxima at 270–285°C and 490–520°C. The maxima of samples dried at 105°C were somewhat shifted towards higher temperatures. The thermal decomposition of myosin starts with endothermic melting and on the DTA* curves there are exothermic maxima in two temperature ranges, from 280 to 320° and from 480 to 535°C. The first exothermic maximum of samples freed from lipids and dried at 105°C is decreased, whereas the second is increased and shifted to higher temperatures.

The myofibril, although it is composed of more components than is myosin, exhibits fewer decomposition rate maxima. Freeze-dried samples showed three, whereas samples dried at 105°C and freed from lipids and taken from E-avitaminotic animals displayed only one maximum.

Maxima indicating the value of exothermic reaction heat could be discerned only with samples freeze-dried or dried at 105°C.

The DTA curves of samples freed from lipids and dried at 105°C are very similar to those of E-avitaminotic myofibril, as well as of E-avitaminotic myofibrils freed from lipids and dried at 105°C. From this observation it may be concluded that the removal of lipids and E-avitaminosis bring about changes of similar character in the thermal behaviour.

Erdey (1968) has surveyed in detail the biochemical and clinical applications of thermoanalytical methods, as well as the complex thermoanalytical method itself. The principle, construction and operation of the apparatus, the derivatograph, was described by Paulik et al. (1958). Substances of various types, thus also organic materials, can be analyzed by the instrument. We used it for the examination of myosin and myofibrils.

The systematic study of glycosaminoglycan derivatives, such as chondroitic sulphate, hyaluronic acid, heparin and β-lipoprotein, was initiated by Bihari-Varga and her co-workers (1968, 1969, 1971), and they established that although these complex materials were the mixtures of hexosamines, uronic acids, sulphate esters and other carbohydrate derivatives, they gave sharp and characteristic DTG peaks.

The DTG maxima of proteins are found in a relatively narrow temperature range different from that of glucosaminoglycans. The thermoanalytical investigation of protein has hardly commenced. Collagen and elastin were studied by

* *Abbreviations used*: TG, thermogravimetric curve; DTG, differential thermogravimetric curve; DTA, differential thermoanalytical curve.

Simon et al. (1968), whereas among the globular proteins cytochrome-c and ribonuclease and among the fibrillar proteins fibrinogen and fibrin were analyzed by Bihari-Varga (1971).

Muscle proteins have not yet been subjected to thermoanalytical investigations. In our experiments we used purified myosin and its chromatographic fractions, as well as myofibril preparations obtained from healthy and vitamin E-deficient muscle.

The properties of myofibrils prepared from vitamin E-deficient muscle, markedly differ from those of normal muscle. In the vitamin E-deficient muscle myofibrillar actomyosin and myosin contents are strongly decreased, striation is blurred, the decomposition of structure becomes more and more pronounced, while the amount of scleroprotein fraction increases (Mason, 1960). In the vitamin E-deficient muscle the activity of lysosomal enzymes is enhanced, especially that of cathepsin, which increases 10 to 15-fold (Zalkin et al., 1962). There is an about 30-fold rise in the metabolic rate of DNA, whereas an only 5-fold rise in RNA. In spite of this the specific incorporation of labelled glycine into the muscle proteins of vitamin E-deficient animals is lower than that with normal animals (Dinning, 1962).

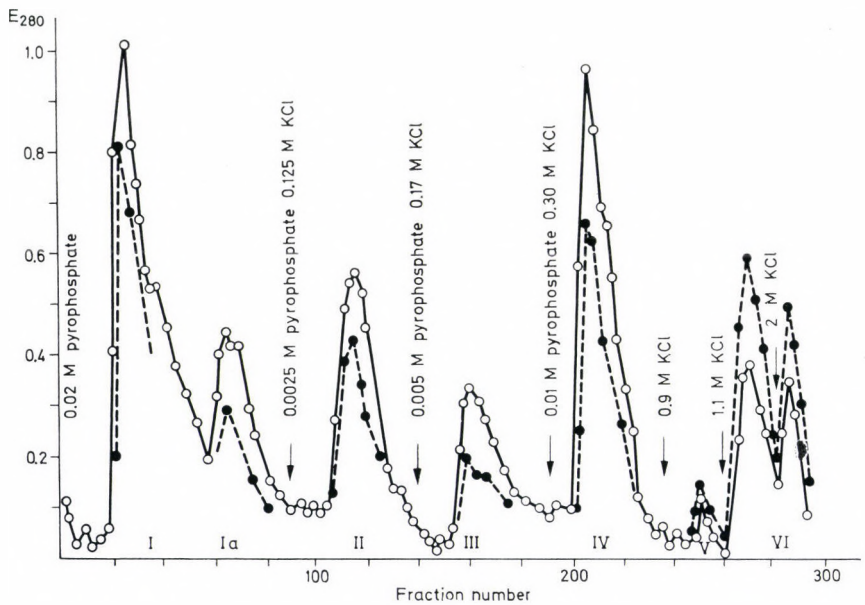

Fig. 1. DEAE-cellulose chromatography of ultracentrifuged myosin. A protein sample, in 0.02 M pyrophosphate buffer pH 7.6 giving an A_{280} value of 311.8 in 1 ml was applied to a column. Column size: 2.3 × 50 cm. The column was equilibrated with 0.01 M pyrophosphate buffer, pH 7.6, containing 0.02 M 2-mercaptoethanol. Fraction volume: 6.2 ml. —, A_{280}; - - -, A_{260}

Materials and methods

Myosin was extracted from rabbit muscle homogenate according to the method of Szent-Györgyi (1951), but the modified extracting solution of Portzehl et al. (1950) was used.

After purification myosin was centrifuged at 105 000 $\times g$ for 1 hour, then dialysed against 0.02 M pyrophosphate buffer, pH 7.2, followed by chromatography on DEAE cellulose column as described by Baril et al. (1967). The chromatographic procedure and the properties of the invidual fractions have previously been described (Fazekas et al., 1971).

Myofibrils were prepared by the method of Perry and Corsi (1958) or of Perry and Zydowo (1959). Since the homogenizing solution contained 5 mM EDTA, we obtained relaxed myofibrils. Myofibrils were used for thermoanalytical studies without treatment, in the freeze-dried state, whereas myosin fractions were analyzed in the freeze-dried state.

The thermoanalytical measurements were carried out in a Paulik – Paulik – Erdey (1958) type MOM or GYEM derivatograph. The instrument simultaneously registers the change of weight (TG), the rate of weight change (DTG), the temperature (T) and the change of enthalpy (DTA) in the same sample. Heated Al_2O_3 served as reference. The samples (about 100 mg) were placed into platinum crucibles. Heating rate was 3 °C per min. The samples were analyzed up to 500 or 600 °C, in an inert (argon) atmosphere. The flow rate of argon was 20 l per hour.

Results

The thermoanalytical curves of myosin

Fig. 1 shows the chromatogram of myosin obtained from mixed (dorsal and hindlegs) muscle. Chromatographic fractions denoted with II, III, IV and V displayed ATPase activity.

Fig. 2 presents the DTG curves of non-chromatographed myosin (fraction 0), myosin fractions II, III and IV, lipid-free myosin and triolein. The first peak on the DTG curves of all five myosin fractions at 70 or 75 °C indicates the loss of water content; around 220 to 230 °C the melting of proteins can probably be observed (lyotropic state), which introduces the thermal decomposition of proteins. The latter attains its highest rate between 270 and 290 °C, depending on the sample. Above this temperature the rate of decomposition decreases, then between 490 and 520 °C it proceeds again with maximal speed, except for the lipid-free samples, which exhibit the maximal rate of decomposition at 555 °C.

Triolein has a DTG curve different from that of proteins, decomposition is confined to a relatively narrow temperature range.

Fig. 3 demonstrates the DTA curves of the above samples. The most details are seen with non-chromatographed myosin (fraction 0), in accordance with its being composed of several components. The least details are found on the DTA

curve of lipid-free myosin. The lower thermal maxima become flat, but the area under the exothermic peak with a maximum at 530 °C increases at the expense of smaller peaks.

The DTA curve of triolein is entirely different from those of myosin samples.

The thermoanalytical curves of myofibrils

The myofibril has a complex structure. The largest protein component of the myofibril is myosin, which constitutes the major part, about 54–55%, of the myofibril (Huxley, Hanson, 1957), whereas the rest is provided by actin, tropomyosin, troponins, α-actinin, β-actinin and the *z*-membrane protein. The remaining few per cents are probably regulatory proteins. It is expected that the thermoanalytical study of the myofibril allows one to draw certain conclusions about the structure of the myofibril.

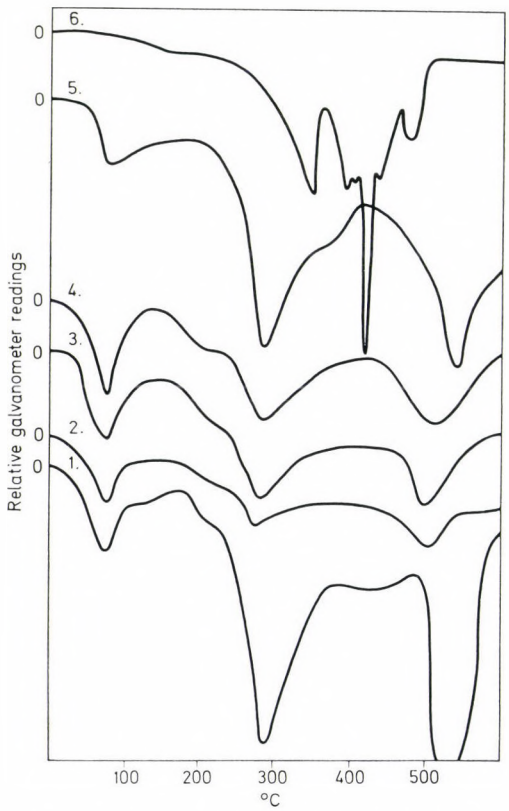

Fig. 2. DTG curves of myosin samples and triolein. 1) Unresolved myosin (fraction 0); 2) myosin II fraction; 3) myosin III fraction; 4) myosin IV fraction; 5) lipid-free myosin; 6) triolein

Fig. 4 shows the DTG curves of the following 5 samples: freeze-dried myofibril, myofibril dried at 105 °C, freeze-dried E-avitaminotic myofibril, lipid-free E-avitaminotic myofibril dried at 105 °C. The peak at 70 °C characteristic of water loss can only be seen on the DTG curve of freeze-dried myofibril, and this is also the most detailed. On the DTG curves of the other myofibril samples only a single fused peak can be discerned. This is presumably due, at least in part, to drying at 105 °C and to the removal of lipids.

Fig. 5 illustrates the DTA curves of myofibril samples shown in Fig. 4. In this case, too, the DTA curve of freeze-dried myofibril displays the most thermal maxima and the peaks suggest mainly exothermic reactions. The DTA curves of the other samples seem to support what we said about the DTG curves, namely that drying at 105 °C leads to the fusion of thermal maxima. Although the DTA maxima of myofibrils dried at 105 °C show a tendency to fuse, the reactions are still of exothermic character.

The removal of lipids and drying at 105 °C alter the shape of DTA curves.

This curve is already very similar to the DTA curve of E-avitaminotic myofibril.

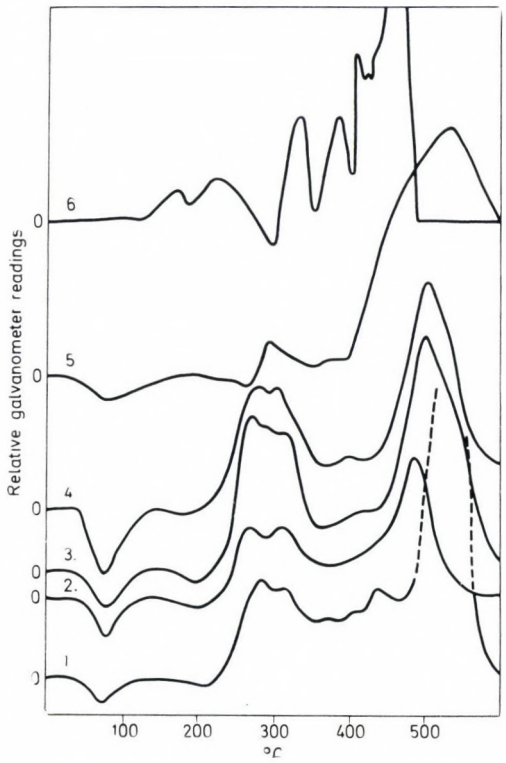

Fig. 3. DTA curves of myosin samples and triolein. Numbering the same as in Fig. 2

Discussion

In the present experiments we studied the thermal behaviour of a contractile protein, myosin and of a complex entity responsible for muscle contraction, the myofibril. Myosin itself can be resolved into several proteins possessing ATPase activity, some accompanying proteins and two lipid-containing fractions (Fig. 1, fractions V and VI). Accordingly, non-resolved myosin (fraction 0) exhibits the most details on the DTG and DTA curves, whereas less detail can be discerned on the curves of lipid-free and separated myosin fractions. The peaks found on the DTA curves of myosin indicate the following processes: the endothermic peak around 70 °C represents dehydration; the peak from 195 to 215 °C means the melting of protein; the 280 to 320 °C peak is the manifestation of the first exothermic decomposition, whereas the one between 490 and 530 °C reflects the major exothermic decomposition.

The first exothermic decomposition has two relatively well resolved DTA peaks, which show a fused profile with lipid-free myosin. The thermal maxima of

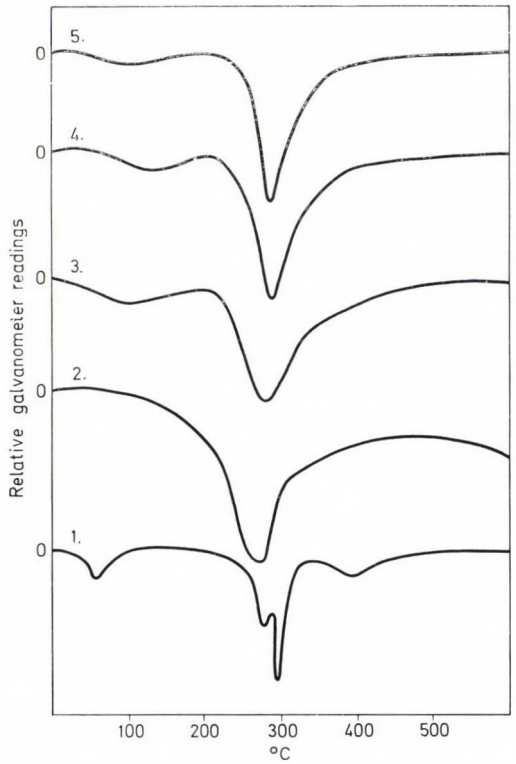

Fig. 4. DTG curves of myofibril samples. 1) Freeze-dried myofibril; 2) myofibril dried at 105°C; 3) myofibril freed from lipids and dried at 105°C; 4) freeze-dried E-avitaminotic myofibril; 5) E-avitaminotic myofibril freed from lipids and dried at 105°C

triolein markedly differ from those of the proteins. However, it is not excluded that the small amount of lipid (mainly phospholipid) present in the myosin preparations should be taken into account when analysing the thermal decomposition curves of myosin.

Among the myofibril samples the DTG and DTA curves of freeze-dried myofibrils do not exhibit more details than expected from the number of components relative to myosin. In myosin the independence of decomposition of the components on the thermoanalytical curves is more pronounced than with the myofibrils.

This fact may be explained by the percentual distribution of components. The decomposition of minor components encountered in the myofibrils in a few per cent, may take place at the same temperature as that of the main component. In fact, the curves indicate that proteins undergo thermal decomposition mainly in two temperature ranges. Furthermore, even if the minor components are decomposed at a different temperature, the overlap of exothermic and endothermic reactions may blur their decomposition peaks. It is probably due to the circumstance that the peaks corresponding to lipids do not prevail in either the DTG or DTA curves

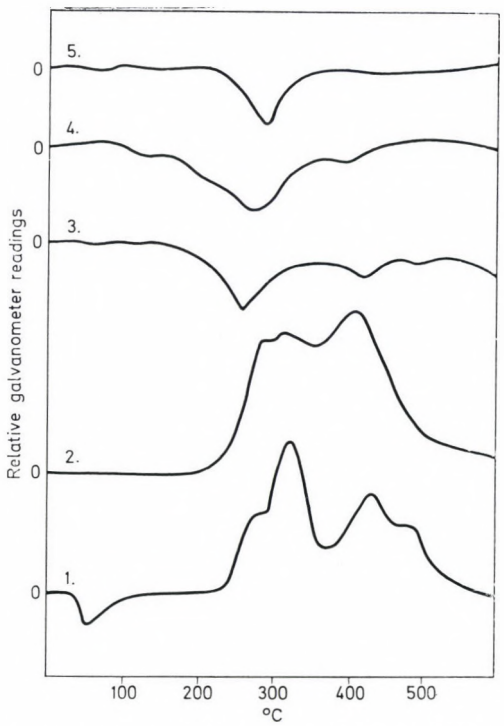

Fig. 5. DTA curves of myofibril samples. Numbering the same as in Fig. 4

Drying at 105 °C results in the fusion of the peaks of DTG and DTA curves. This can presumably be explained by secondary reactions caused by drying for several hours, as a consequence of which unresolved myosin and myofibrils show the signs of structural disintegration. The fusion of peaks could also be explained by the great changes in the composition of myosin and myofibril elicited by high temperature, as a result of which both systems exhibited a behaviour on the thermograms characteristic of homogeneous substances consisting of a single component. This observation was more pronounced with lipid-free proteins dried at 105 °C. About 50 to 70% of myosin content and about 20 to 40% of actin content are lacking from E-avitaminotic myofibrils. The deficient myofibril consists of about 10–20% connective tissue protein and at least 30 other components. Its thermal decomposition curves resemble, even in the freeze-dried state, the DTG and DTA curves of samples freed from lipids or dried at 105 °C.

Our experiments clearly indicate that thermoanalytical studies should be performed with highly purified protein preparations in order to be able to make calculations from the DTG and DTA curves and to make meaningful comparisons with regard to the bonds being decomposed.

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A Simple Method for ATPD etermination in Bacteriophage T4

(Short Communication)

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ATP has been found associated with the tail protein of normal T-even bacteriophages. When a phage particle interacts with the host cell, contraction occurs with concomitant hydrolysis of nucleoside triphosphates (Kozloff, Lute, 1959). The contraction of the tail protein of the T-even bacteriophages seems to be similar to that of actomyosin. This resemblance has suggested that nucleoside triphosphates might play an important role in the contraction mechanism of the tail sheath. Sarkar et al. (1964) have found that the addition of ATP to a purified preparation of contracted sheaths results in their elongation to a certain extent.

To elucidate the role of ATP in the contraction mechanism of bacteriophages makes necessary to have a simple and sensitive method for the determination of ATP associated with the tail protein. ATP and other nucleotides have usually been extracted from T-even bacteriophages with perchloric acid (Wahl, Kozloff, 1962) or trichloroacetic acid (Kozloff, Lute, 1959). Now we have developed a simple method for ATP extraction from bacteriophage T4 by heat treatment. This procedure in the case of other dissociated systems, i.e. different cultured cells, has been proved to be very advantageous (Kalbhen, Koch, 1967; Grimm, Frank, 1972).

Cultures of *E. coli* B – grown in M9 synthetic medium (Adams, 1959) under vigorous aeration to a concentration of 2 to 3×10^8 cells/ml at 37 °C – were infected with 3 phage particles/cell. Following 4 hours of aeration the cells were lysed by the addition of chloroform. Such lysates yielded a phage titre of 1 to 4×10^{11} pfu/ml. After removing the bacterial debris by centrifugation phages were concentrated by precipitation at their isoelectric point, pH 3.9. The collected precipitates were dissolved in Tris-HCl buffer (pH 7.0). By the addition of a small amount of crystalline deoxyribonuclease (1 μg/ml) the phage preparation was freed from bacterial DNA. The remaining insoluble material was removed by low speed centrifugation. For further purification and concentration the phage particles were sedimented by centrifuging them at $30\,000 \times g$ for 30 minutes. The pellet obtained was allowed to leach out in 0.06 M Tris-HCl buffer (pH 7.0). Finally, to remove any retained impurity the suspension of phage particles was centrifuged at low speed. The clean, in UV-light fluorescent phage stocks contained 1.65×10^{13} pfu/ml with a protein content of 8.4 mg/ml, as determined according to the method of Lowry et al. (1951).

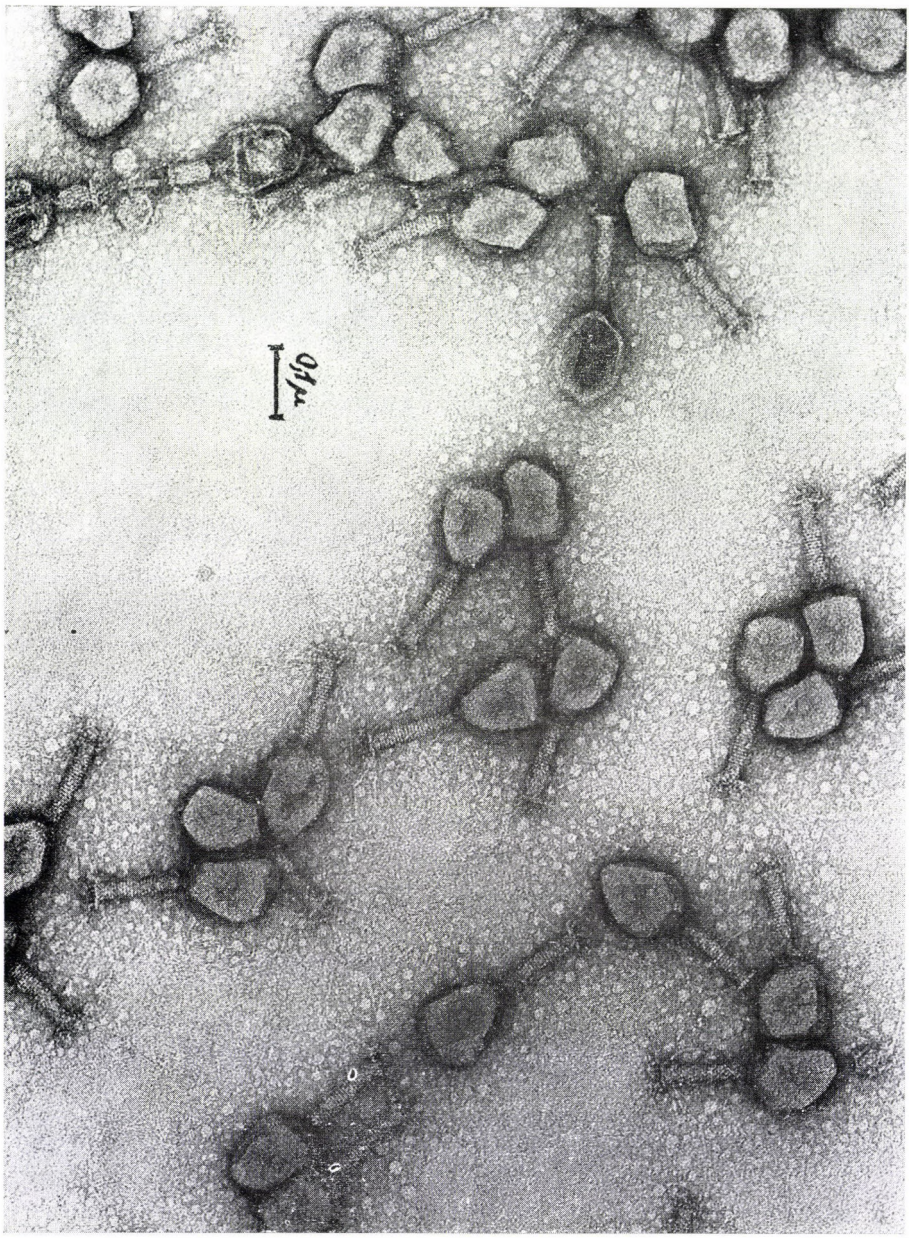

Fig. 1. Electron micrograph of T4 bacteriophages. Samples were negatively stained with uranyl acetate and examined in a JEM 100 B electron microscope

For extraction of ATP from bacteriophage T4 0.9 ml bidistilled water was added to 0.1 ml phage stock. Then the test tubes were placed in a hot water bath for the time required. After one, five and fifteen minutes the extraction procedure was stopped by placing the samples in an ice bath. This was followed by centrifugation at $5000 \times g$ for 30 minutes. The supernatant fluid was used for ATP determination carried out according to the method of Strehler and Totter (1953) on a Packard Tri-carb Liquid Scintillation Spectrometer. The firefly lantern extract (luciferin-luciferase-system) and the adenosine-5'triphosphoric acid disodium salt (ATP) were purchased from SERVA (Heidelberg) and REANAL (Budapest), respectively.

Table 1

Extraction of ATP from bacteriophage T4 by heat treatment

The mean errors and the number of the experiments (in brackets) are also shown in the first column

Duration of heat-treatment, (minutes)	Counts $\times 10^{-3}$ per 0.2 min produced by T4 bacteriophage equivalent to 1.65×10^{11} virus particles	ATP content, nmoles per T4 particle	Number of ATP molecules per phage particle
1	48 ± 4 (10)	1.9×10^{-13}	114
5	44 ± 4 (11)	1.8×10^{-13}	108
15	37 ± 3 (8)	1.7×10^{-13}	102

It has been accepted by many authors that the ATP found in T-even bacteriophages is not an artifact resulting from contaminating bacterial debris. In spite of this we checked the purity of the different phage preparations both by a series of electron micrographs taken at different magnifications and by the determination of the protein content. Fig. 1 shows a medium-sized magnification EM picture of bacteriophage T4. This electron micrograph represents a typical EM picture of our phage stocks. Values for ATP extracted by one, five and fifteen minutes heat-treatment are given in Table 1. The results in the first column represent the average values of the light emission expressed as counts $\times 10^{-3}$/0.2 min. The corresponding ATP values were obtained from our ATP standard curve, which was linear in the range of measurements. The ATP contents of the phage samples treated with heat for different time are shown in the second and third column of Table 1. The results are expressed as nmoles of ATP and as molecules of ATP per T4 phage particle.

According to Kozloff and Lute (1959) the ATP content of intact T2 phage after extraction with cold trichloroacetic acid was about 87 molecules per T2 particle. When before extraction the protein fibres at the tip of the tail were removed they determined 135 molecules of ATP per T2 particle. It appears that 35% of ATP + dATP is bound deep inside the tail structure of intact T2 phage and cannot be extracted with cold acid because of occlusion.

As our results show heat treatment is an effective procedure for the extraction of ATP in concentrated phage suspensions. We determined 114 molecules of ATP per T4 particle after a one-minute heat treatment. Thus heat extraction is an easier and quicker method than perchloric acid extraction.

The authors wish to thank Dr M. Guba-Mészáros for taking the excellent EM pictures, Mr M. Huber for his help in ATP determinations and Mrs G. Papp for her skilled technical assistance.

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On the Nature of Frequency Dependence of the Evoked Potentials in the Cat's Auditory Cortex

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A model is proposed by the authors for the transmitter dynamics of the thalamocortical synapses. The transmitter store is represented within this by a condenser which is charged by a voltage source, corresponding to transmitter synthesis, through a resistor. The transmitter quantities liberated by nerve impulses are represented by electric charges flowing through the discharging resistor and a switch shortcircuiting the condenser. At properly chosen parameters the amounts of charges leaving the condenser at subsequent shortcircuits vary in an analogous manner as do the transmitter quantities liberated by nerve impulses. The frequency dependence of the acoustically evoked potentials led from the cat's cerebral cortex can be adequately explained in this way.

Introduction

In the course of our previous studies we examined how the amplitudes of the potentials evoked by acoustic and medial geniculate body (MGB) stimulation depended on the stimulus repetition rate in the acoustic cortex of anaesthetized cats. Our first intention was to answer the question whether the decrease in amplitude going along with increasing stimulus frequencies covers the criteria of habituation (Fehér, 1973). In those experiments it turned out that this frequency dependence meets only a part of the criteria of habituation formulated by Thompson and Spencer (1966). Summing up all evidences we concluded that with all probability the phenomenon might be attributed to synaptic depression due to the depletion of transmitter stores. A model has been derived from these assumptions to describe the transmitter dynamics of the thalamocortical afferent terminals. The functioning of this model has been simulated on a computer. We compared the curves obtained in this way with those derived from experimental results and drew some conclusions as to the mechanism of the frequency dependence.

Methods

Adult cats of both sexes were anaesthetized with sodium pentobarbital (40 mg/kg, Synthetic) or with chloralose (80 mg/kg, Light and Co.). After the skin and muscles were removed, the skull was opened and the cortex exposed over the g. ectosylvius. The head of the animal was fixed in a stereotaxic apparatus. A small ear-phone was inserted into the right ear of the animal and via this supramaximal

click stimuli were presented from a square wave stimulator. In other experiments the left MGB was stimulated stereotaxically by impulses of the following parameters: duration 0.3–0.5 msec, amplitude 8 to 14 Volts. The co-ordinates were taken from the stereotaxic atlas by Jasper and Ajmone-Marsan (1960).

The potentials evoked in the afore-mentioned ways were led off from the primary auditory area (AI) of the g. ectosylvius anterior. The leads were taken from a depth of 1500 to 1800 microns. The focus of origin of the evoked potentials can be localized at this depth. Records taken from here are not distorted by the volume conductor of the cortex. For stimulation, amplification and recording we used a DISA two-channel Electromyograph with a Cossor Camera.

A sequence of evoked potentials consisting of 20 to 100 samples was recorded at each frequency either photographically or on a magnetic tape. The usual sequence of stimulus parameters was 0.1; 0.2; 0.5; 1.0; 2.0; 5.0; 10.0 cps. The photographic records were then projected, and the amplitudes of the last 20 to 25 potentials within each sequence, exhibiting constant level, were measured at standard magnification. From these data averages and standard deviations were calculated. Data stored on magnetic tape were displayed on a NTA 512-channel amplitude analyser making use of an analog converter. The summed curves of 20 to 100 potentials were then plotted by a two-co-ordinate potentiometer on recording paper.

Frequency dependence curves were obtained by plotting the average amplitudes versus the logarithm of the time separating the stimuli at different frequencies.

Results

Upon the presentation of rhythmic acoustic clicks or electric MGB stimuli, the amplitudes of the evoked potentials show a characteristic exponential fall. The experimental data are shown in Fig. 1. The tendency of the amplitude change, though somewhat masked by the biological variability, is quite apparent at 5–10 cps. The amplitudes seem to approach to a limit asymptotically, which well characterizes what happens at the given frequency. The variability of responses was the less the deeper the anaesthesia.

If the limit values obtained in these experiments are plotted against the logarithm of the time separating the stimuli one can plot a curve of frequency dependence. The overall picture of these curves may be characterized by a horizontal, nearly linear, section at long time intervals (low frequencies) and a rather steeply decaying section at short intervals (high frequencies). The inflexion point of these two sections is between $t = \log 0.5$ and $\log 3.0$ sec. Another, but less distinct, inflexion point appears at high frequencies, i.e. at $\log t = 0.10-0.14$ sec (Fig. 2).

When 1 per cent strychnine solution was applied to the cortex, the strychnine potentials exhibited a frequency dependence essentially similar to that of Fig. 2, but more closely piece-wise linear (see Fig. 3). Since strychnine depresses – at least

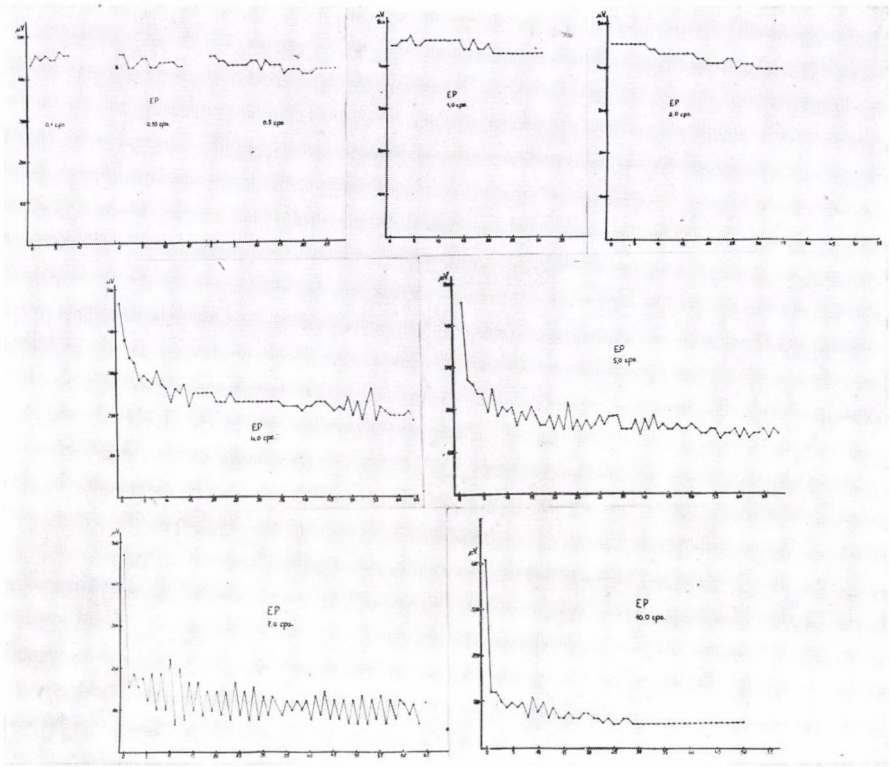

Fig. 1. Amplitudes of the acoustically evoked potentials (in microvolts) at rhythmic stimulation of different frequencies (ordinates). Abscissae: serial number of impulses. (Frequency of stimulation is given in every diagram separately)

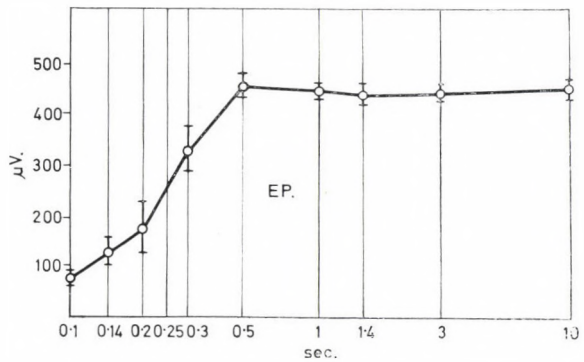

Fig. 2a. Frequency dependence curve of acoustically evoked potentials and MGB potentials (Fig. 2b). Ordinates: amplitudes in microvolts. Abscissae: logarithmic values of the times separating the stimuli at different frequencies

partially – cortical inhibitions, one cannot ascribe any major role to inhibition in cortical frequency dependence. Therefore, in modelling cortical processes, we preferred the dynamics of transmitter liberation and supply, for which we started from the following assumptions:

1. The transmitter liberated from the thalamocortical nerve endings elicits postsynaptic potentials on the membrane of the neurons in layers III and IV. The evoked potential, as recorded, is the sum of these postsynaptic potentials (Bremer, 1958).

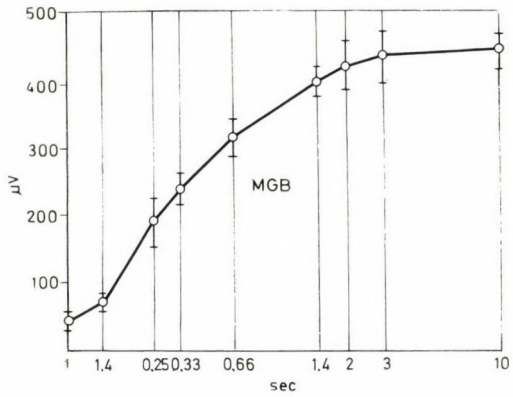

Fig. 2b.

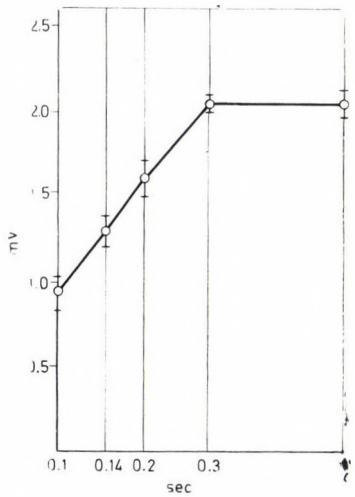

Fig. 3. Frequency dependence curve of strychnine potentials. (Notations as in Fig. 2)

2. The amplitude of the postsynaptic potentials, as well as that of the evoked potentials is in some way proportional to the local concentration of the transmitter bringing it about (Curtis, Eccles, 1960).

3. Upon the effect of a single impulse always the same proportion of the actual transmitter store is released (Perry, 1953).

4. The transmitter store is replenished at a rate depending on the actual pool.

5. The duration of excitation at the terminals, releasing the transmitter, is always the same.

Considering these assumptions we arrived at the following model (Fig. 4).

Condenser C is charged through resistance R_1 by the voltage source B with voltage V_0. At some instant the switch S is closed for 2 msec and the con-

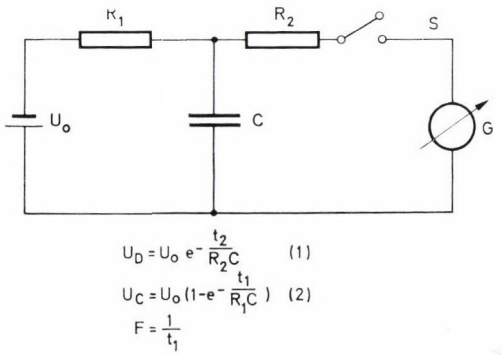

Fig. 4. Model of transmitter dynamics and the equations describing its function. (For explanation see the text)

denser C is discharged through resistance R_2. Let us assume, for the moment, that charging of condenser C is during the discharging cycle negligible. Within the intervals between the discharges the condenser is being charged. The momentary voltage of the condenser depends on the time constant (R_1C) of the charging and that of the discharging circuit (R_2C) as well as on the intervals between the discharges (t_1) and the duration of the discharges (t_2). According to this, the condenser voltage is at the end of the first discharging cycle

$$V_D \approx V_0 e^{-\frac{t_2}{R_2C}} \tag{1}$$

and after the next charging cycle

$$V_C = V_0\left(1 - e^{\frac{t_1}{R_1C}}\right). \tag{2}$$

It is easy to realize that if the time constant of the charging circuit, T_1, is longer than that of the discharging one, T_2, complete recharging is only possible if the time for charging t_1 is as many times longer than t_2, as many times T_1 exceeds T_2.

Since during repetitive stimulation the amplitude of the evoked potentials shows a progressive decline, one is led to consider that the model will be adequate if T_1 is much higher than T_2. This means for the model, that the condenser voltage

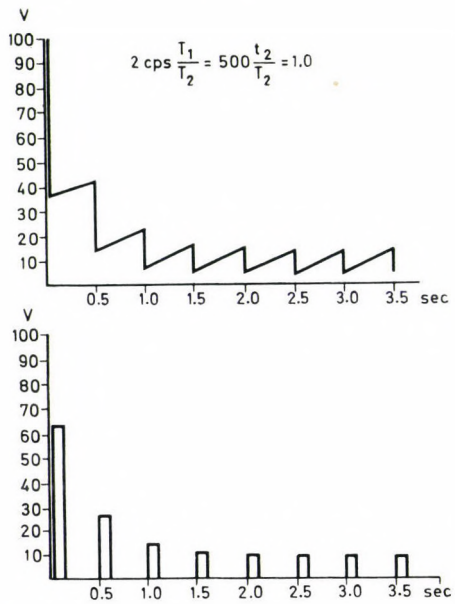

Fig. 5. The mode of functioning of the model presented in Fig. 4. Upper diagram: changes in the tension of condenser C in the course of rhythmic closing of switch S at parameters given in this Figure. Lower diagram: charge quantities leaving the condenser at subsequent discharges. These tend to a well defined limiting value. Ordinates: tension in arbitrary units. Abscissae: time in milliseconds

remains lower after each charging cycle than it was after the previous one. From the exponential character of Equations (1) and (2) one can predict that the voltages appearing after successive charging cycles will differ from one another less and less, and the charge quantities leaving the condenser tend to some limit. This is illustrated in Fig. 5, in which the upper diagram represents the change of the condenser voltage during a uniform and rhythmic series of discharges. Since this decrease of voltage is proportional to the amount of charges leaving the condenser, it can be regarded as the analogue of the pulsatory transmitter release. In this example $\frac{T_1}{T_2}$ is taken for 500, the frequency for 2 cps and the ratio $\frac{t_2}{T_2}$ for

1.0. A comparison of this diagram and the experimental curves reveals that the processes underlying them may have common features.

If we take as characteristic value for a given stimulus frequency the ordinate of the horizontal section following the previous abrupt fall, the question arises *a*) under what conditions does the condenser voltage really approach to a limit and, if it does, *b*) do the limiting values obtained at different frequencies show a dependence on the logarithm of times separating the stimuli, just as they do in actual experiments.

Concerning question *a*) the following considerations may be made.

In case of long-lasting closure of the switch the condenser voltage takes the value,

$$V_2 = V_0 \frac{R_2}{R_1 + R_2} . \tag{3}$$

Let the voltage of the condenser be equal with $V_{C(i)}$ at the end of the i-th charging cycle and $V_{D(i)}$ at the end of the i-th discharging cycle. Let t_1 be the duration of the charging cycle and t_2 that of the discharging cycle. Let T_1 be the time constant of the charging circuit and T_2 that of the discharging circuit. We take the presence of B and R_1 during the discharging period also into account. Therefore, $T_1 = R_1 C$ and $T_2 = C \frac{R_1 R_2}{R_1 + R_2} \simeq R_2 C$.

Then

$$V_{D(i)} = V_2 + (V_{C(i-1)} - V_2)\ \mathrm{e}^{-\frac{t_2}{T_2}} \tag{4}$$

$$V_{C(i)} = V_{D(i)} + (V_0 - V_{D(i)})\ \left(1 - \mathrm{e}^{\frac{t_1}{T_1}}\right) . \tag{5}$$

After subsequent discharges and charges the voltage of the condenser approaches to a limit and after the n-th charge it equals with

$$\lim V_{C(n)} = V_{C(0)} \lim \alpha^{n+1} + K \lim \frac{1 - \alpha^n}{1 - \alpha} \tag{6}$$

$$n \to \infty ,$$

if $\alpha < 1$, then

$$\lim V_{C(n)} = K \frac{1}{1 - \alpha} \tag{7}$$

$$n \to \infty .$$

Here

$$\alpha = \mathrm{e}^{-\frac{t_2}{T_2} - \frac{t_1}{T_1}} < 1 \tag{8}$$

and

$$K = V_2(1 - e^{-\frac{t_2}{T_2}})\, e^{-\frac{t_1}{T_1}} + V_0(1 - e^{-\frac{t_1}{T_1}}) . \tag{9}$$

Accordingly, the condenser voltage approaches after the n-th discharge to

$$\lim_{n \to \infty} V_{D(n)} = K \frac{e^{-\frac{t_2}{T_2}}}{1 - \alpha} + V_2(1 - e^{-\frac{t_2}{T_2}}), \tag{10}$$

The amount of charges leaving the condenser at the n-th discharging cycle has, of course, also a limit but its algebraic expression is here, for sake of brevity, omitted.

Adopting the model just described in detail computations have been made.

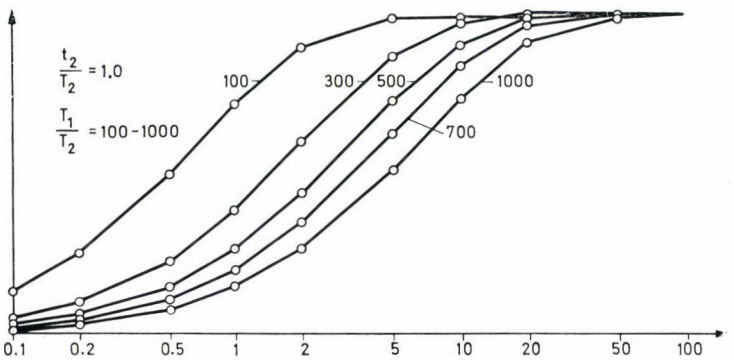

Fig. 6. Computed frequency dependence curves at parameters given in the Figure

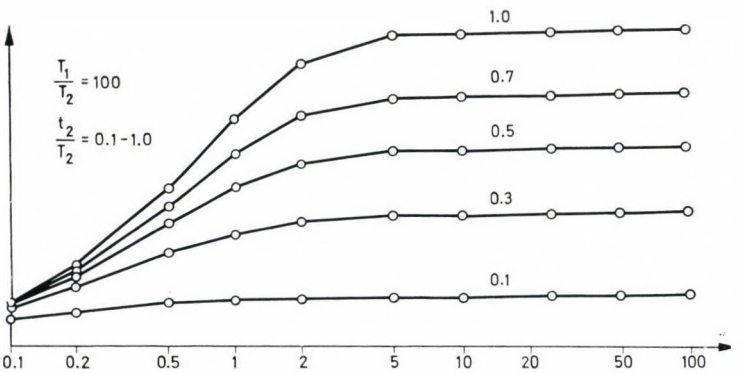

Fig. 7. Computed frequency dependence curves at parameters given in the Figure

Three parameters were chosen to be varied. The ratios $\frac{T_1}{T_2}$ were changed by 100 steps from 100 to 1000. The ratio of the discharge time to the time constant of the discharging circuit, $\frac{t_2}{T_2}$ was varied by 0.1 steps from 0.1 to 1.0. The time intervals separating the stimuli ranged from 0.1 to 100 seconds.

A family of curves obtained by the computer are shown in Figs 6 and 7. In Fig. 6 $\frac{t_2}{T_2}$ was kept at 1.0 and $\frac{T_1}{T_2}$ was varied between 100 and 1000. It may be seen that, by increasing this latter ratio, the boundary point moves toward longer interstimulus intervals.

As a condenser requires for complete charging a time which amounts about five time constants, it is obvious that the abscissa of the upper boundary point equals about the five-fold of the charging time constant. This enables us to estimate the time constant of the transmitter supply and examine the factors acting upon it even though the ratio $\frac{T_1}{T_2}$ is unknown. Keeping $\frac{T_1}{T_2}$ constant and ranging $\frac{t_2}{T_2}$ from 0.1 to 1.0 a family of curves shown in Fig. 7 has been obtained. As $\frac{T_1}{T_2}$ is kept constant the location of the boundary point remains unchanged but at the same time the height of the horizontal section rises and the slope of the falling section increases. This latter can be expressed by the slope of the straight line between the boundary point and the ordinate value at 0.1 sec. On the abscissa of the calculated curves this means a span of two orders of magnitude. If the model is expected to be pertinent to the cortical processes, from the slopes of the experimental curves interpreted in this way, one can estimate the ratio $\frac{t_2}{T_2}$. Fig. 8 shows the correlation between the slope of the frequency dependence curve and the ratio $\frac{t_2}{T_2}$. Assuming that the ordinates were obtained by dividing the amplitudes of the boundary point by the amplitude measured at a time less than two decimal orders of magnitude, one may estimate in this way $\frac{t_2}{T_2}$ from the experimental curves, if the boundary point has already been found and the curve is known over two orders of magnitude, toward high frequencies.

If the frequency dependence curve does not span two orders of magnitude from the boundary point toward high frequencies, one can still estimate $\frac{t_2}{T_2}$ by another approach.

As it can be seen from Fig. 9 the relative difference between the first and second sample of a potential sequence at 10 cps frequency scarcely depends on $\frac{T_1}{T_2}$ and is essentially a function of $\frac{t_2}{T_2}$.

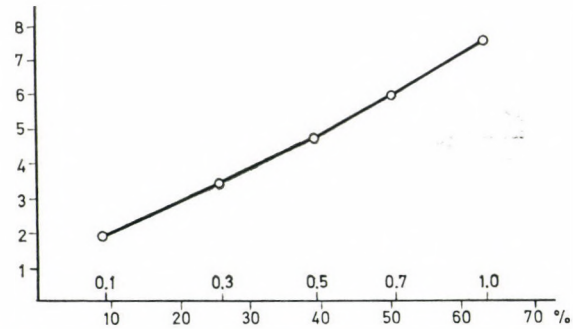

Fig. 8. The ratio of amplitudes at the boundary point and at a time less by two decimal orders of magnitude. Abscissa: the proportion of the transmitter pool which is liberated by an impulse (per cent scale) and the values of $\frac{t_2}{T_2}$ (upper scale)

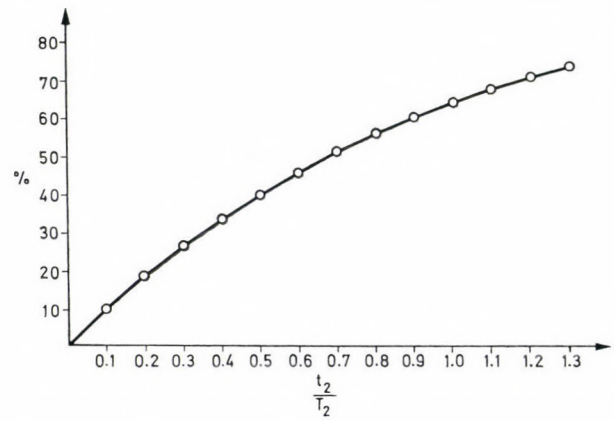

Fig. 9. The percentual difference of the 1st and 2nd impulse (in per cent of the 1st) at 10 cps (ordinates) and the values of $\frac{t_2}{T_2}$ belonging to it (abscissa)

If, now, one records the first two samples of a potential sequence at 10 cps and computes the ratio of them (first/second amplitude) one immediately obtains the value of $\frac{t_2}{T_2}$ from the diagram shown in Fig. 9.

If the source voltage is increased, all other voltages increase to the same extent and so do also the voltage changes due to the discharges of the condenser. However, the position of the boundary point remains unchanged.

Two questions may be now raised: *a*) how can one determine the parameters of the model from the experimental curves (viz. T_1, T_2, t_2, $\frac{t_2}{T_2}$), and if once

determined, *b*) how to explain the results and how to derive from all these data information on the cortical neural processes?

From estimating the parameters of the frequency dependence curves obtained from experiments with click-evoked and MGB potentials, we obtained the following results.

The curve of the MGB potentials (diagram of Fig. 2b) shows a boundary point at 10 sec, and T_1 approximately equals 2 seconds. T_2 is unknown and cannot be determined from the experimental data. Adopting, however, the aforementioned approach the ratio $\frac{t_2}{T_2}$ can be calculated in two ways. This ratio indicates what proportion of the transmitter store is liberated by a single impulse and may be therefore associated with the permeability of the nerve membrane to the transmitter. Assuming that the duration t_2 of discharges is 2 msec, being analogous with the duration of the spike potential during which the transmitter is liberated, one can readily estimate the value of T_2 from $\frac{t_2}{T_2}$. For MGB potentials $\frac{t_2}{T_2}$ equals 0.6, that means that about 50 per cent of the actual transmitter store is liberated upon the effect of an impulse. For T_2 we get the approximate value of 3 msec and the ratio $\frac{T_1}{T_2}$ may be about 600.

In the curve of the evoked potentials (diagram of Fig. 2a) the boundary point is at 500 msec; thus T_1 is about 100 msec. The ratio $\frac{t_2}{T_2}$ can be determined from the percentage difference of the first and second potential samples of the 10 cps series. The respective amplitudes are 396 μV and 119 μV. Their difference is 70 per cent as related to the first one. According to Fig. 9 this corresponds to a $\frac{t_2}{T_2}$ ratio of 1.2. This means that an impulse empties 70 per cent of the transmitter store. Taking t_2 for 2 msec again, we obtain 1.7 msec for T_2 and 60 for $\frac{T_1}{T_2}$. This considerably differs from the analogous value calculated for the MGB potentials but one has to take into account, that the curves were obtained from two different experiments.

Discussion

The model described in this paper was obtained by using several relevant simplifying assumptions. It is of no doubt, that simplifications of this sort are hardly applicable for single neurons or reflex arcs but may be practicable for large neuron populations which, under constant physiological conditions, may well underlie statistical generalizations. This particularly holds for assumptions 2 and 5, which postulate the relative stability of the excitability and permeability of the pre- and postsynaptic membranes at least for the period of the experiment.

The value of a model depends on how fundamental are the simulated processes and how faithfully are they analogized by the model. A model is expected to predict the behaviour of the system under conditions similar to those existing in the biological object.

Crucial for this correspondence is, whether the parameters of the model can really be interpreted in the biological system and the changes in its function can be really expressed as consequences of changes of its measurable parameters.

The charging time constant T_1 of the condenser corresponds to the speed of the transmitter synthesis. V_0 corresponds to the charge of the transmitter pool at rest, V to the actual charge during functioning. Each factor, influencing the speed of transmitter synthesis, will change the value of T_1, which is experimentally measurable. The time constant T_2 of the transmitter liberation cannot be measured experimentally. t_2 represents the duration of the condenser discharge and corresponds to the axonal spike potential which triggers transmitter liberation. Intracellular measurements revealed for this duration values about 2 msec. The ratio $\frac{t_2}{T_2}$ corresponds to that proportion of the transmitter pool which is released by an impulse from the terminal. Factors influencing the transmitter release (permeability of the presynaptic membrane, height of the spike potential) exert their action upon this parameter. If t_2 is estimated as 2 msec T_2 can be calculated from ratio $\frac{t_2}{T_2}$.

At appropriate level of anaesthesia one can conveniently estimate the main parameters of the transmitter dynamics in the cat's cerebral cortex. One can detect in this way all the factors which influence the parameters of this dynamics and modify cortical functions.

The examination of the frequency dependence of evoked potentials gives an insight specifically into the activity of the synapses between the thalamocortical afferents and the pyramidal cells of layers IV and V enables the synaptological examination of them. Of course, the usefulness of the model presented here can be only judged by further investigations of the factors modifying evoked cortical potentials.

A model of this type has previously been devised by Kruckenberg and Sandweg (1968) specifically for the neuromuscular junction.

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Forces Acting Between Muscle Filaments, II. A Theoretical Computation of the Resting Elasticity Curve

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A theoretical attempt is presented to interpret the length-tension curve of resting muscle based on assumed forces between myosin and actin filaments depending on the sarcomere length and side spacing. In this model the assumed role of connective tissue and other parallel elements has been neglected. Taking the elementary force acting between two infinitesimally long parts of myosin and actin in the form $(A/\rho^a) + + (B/\rho^b)$ (A and B are constants, ρ is the distance and a and b are integers) using least squares we could fit the experimental curve very well with an optimum at $a = 7$ and $b = 15$. This result indicates that the length-tension curve of resting muscle may be described theoretically by muscle substance even preserving all the basic assumptions of the sliding model.

Introduction

In the first paper of this series (Garamvölgyi, 1972) we have demonstrated that the filaments of the bee wing muscle do not behave as a body of constant volume, since the decrease of the filament lattice spacing in the course of passive stretch is much greater than required by the constancy of volume. We attributed the different behaviour of vertebrate and insect muscle fibrils to the different lattice geometry. In vertebrate muscles the actin filaments are located in a trigonal position in the centres of the triangles formed by three myosin filaments (Hanson, Huxley, 1955). In these muscles the filament lattice seems to change in such a manner that the volume of the sarcomere remains virtually constant (Huxley, 1952; Elliot et al., 1967; Huxley, 1969). Contrary to this, in the bee flight muscle the actin filaments occupy a position at mid-distance between two myosin filaments (Hanson, Huxley, 1955). In this muscle the sarcomere volume is far from constant. This is why we attributed the control of the filament lattice spacing to the force balance existing within the myofibril depending on the length, i.e. on the degree of the overlap.

The model of the sliding filaments is able to explain the curve of the active force development in terms of the number of the possible myosin-actin interacting sites, i.e., as a function of the degree of overlap (Gordon et al., 1964). The length-tension curve of the inactive muscle seems, however, to represent an apparent

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difficulty for this model. The form of the curve is nearly exponential (e.g. Ernst, 1963). As a consequence, simultaneously with the steep increase of the tension the amount of the overlap decreases when the muscle is subject to passive stretch. Near the resting length the tension of the inactive muscle is in fact very low and just near this length the overlap of the two kinds of filaments is close to total (Page, Huxley, 1963). Perhaps it is due to this contradiction that nowadays even the term "muscle elasticity" is missing from the literature (Ernst, 1970). The elasticity of the resting muscle is attributed exclusively to extrafibrillar structures, i.e. to the sarcolemma, cell membrane and sarcoplasmic reticulum (Huxley, 1964). According to Huxley (1969) the actin filaments may move freely alongside the myosin filaments on cushions of long-range forces. This means that the contractile substance itself was nearly ideally inelastic in its inactive state.

This cannot be said in the case of the flight muscle of the insects, in which there are neither connective tissue elements, nor a considerable amount of sarcoplasmic reticulum. Thus the mechanical properties are to be ascribed to the contractile system alone. In accordance with Pringle (1967) we explained the resting elasticity of this muscle by continuations of the myosin filaments anchored in the Z-lines (Garamvölgyi, Belágyi, 1968; Garamvölgyi, 1969). No long-range forces have been taken into consideration.

In the meantime the length-dependence of the filament lattice spacing has been studied extensively by Elliott and Rome (Elliott, Rome, 1967; Elliott, 1967, 1968; Rome, 1967, 1969). Nevertheless, they accounted with one set of cylindrical filaments only and the changing overlap was not taken into account.

On the other hand, we obtained evidence on vertebrate smooth muscle (Garamvölgyi et al., 1971) that even in relaxed muscle a mechanical force is exerted on the myosin filaments.

On the basis of these data, as well as of some other observations of minor importance we (Garamvölgyi, 1970) arrived at the conclusion that it is unnecessary to neglect the muscle substance in relation to the elastic properties of the resting muscle, if there is a theoretical possibility to explain the resting elasticity by means of the structural basis required by the sliding model. The solution of the paradoxical assumption of a decreasing overlap simultaneously with a steeply (exponentially) emerging tension seems to be rendered possible by means of the simultaneous decrease of the lattice constant. This problem is essentially analogous to the change of capacity of a plate condenser the effective surface of which decreases with the decrease of the distance of the plates. The capacity can exhibit an increasing tendency, in spite of the decrease of surface, if the capacity increase caused by the decrease of the plate distance (changing proportionally to the square of the distance) overcompensates the capacity decrease due to the decrease of the effective surface of the condenser. If this were analogously possible in relation to the length-dependence of the resting tension, the sarcomere as a functional unit, would represent a system in which the balance of transverse forces would be in the state of equilibrium at each length with a certain axial stretch force equal to, in opposite sense, the resting tension at a given length.

It is to be considered, how transverse forces acting in a two-filament system of regular crystalline arrangement may result in a longitudinal force. This question can be formulated by other words, whether or not these transverse forces may influence the relative sliding movement of the two kinds of filaments when they are forced to slide past by an external stretch force.

Assumptions and simplifications

In the course of our considerations we postulated that:

1. The length of neither kind of filaments changes when the muscle will be stretched.[1] As numerical length values of filaments we accepted 1 μ for the actin filament and 1.6 μ for the myosin filament (Page, Huxley, 1963).

2. Stretch does not cause any activation of the cross-bridges and the shape of the filaments is exactly cylindrical.

3. The distance between the same type of filaments (d) changes in inverse atio to the square root of the sarcomere length (S), i.e.,

$$S_0 d_0^2 = S \,.\, d^2 = K \,. \tag{1}$$

4. In the course of the calculations we have taken into account only the centre-to-centre distances. Thus neither the real diameters nor the surfaces of the filaments have been considered. (This will be done in the next paper of this series.) This means that the filaments were regarded strictly as lines.

5. Neither the ionic concentration nor the pH will considerably change in the course of stretch.

6. Both kinds of filaments remain exactly parallel in the course of length changes. Neither the Z- nor the M-line influence the change of d.

7. The entire elasticity of the muscle is to be attributed to the myofibrils. Neither the sarcolemma and cell membrane, nor the sarcoplasmic reticulum nor any kind of "other filaments" (apart from the interdigitating discontinuous system of myosin and actin filaments) play any considerable part.

8. All sarcomeres of the muscles are of the same length at a given length of the muscle. At the slack length (equilibrium length) the actin filaments totally overlap the whole length of the myosin filaments, and the two sets of actin filaments penetrating from the two opposite sides just meet at the centre of the sarcomere (Gordon et al., 1964). The sarcomere length has been correspondingly considered as 2.1 μ. The resting length has been accepted to correspond to a sarcomere length of 2.3 μ (total overlap of all cross-bridges). Correspondingly the overlap ceases at a sarcomere length of 3.6 μ. (This is the length of "no-overlap".) At this length the

[1] This assumption does not influence our earlier statements that beyond the upper limit of the range of the changing overlap (i.e., beyond the calculated no-overlap-point) in bee wing muscle the myosin filaments (Belágyi, Garamvölgyi, 1968; Garamvölgyi, 1969), in human striated muscle the actin filaments (Garamvölgyi, 1970) stretch.

active force development is assumed to reach zero (Gordon et al., 1964). The usual length-tension diagram of the muscle has been arbitrarily transformed in this way into a "sarcomere length-tension" curve in agreement with the assumptions of the group of A. F. Huxley (Gordon et al., 1964).

9. As an experimental curve (related to a sarcomere length scale as described above) a curve taken from a frog sartorius muscle shown by Ernst (1963) has been chosen as basis. The form of this curve is nearly exponential (see also Tigyi, 1955 and Fig. 1).

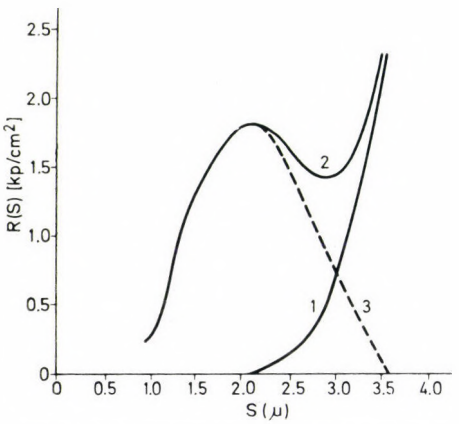

Fig. 1. Length-tension diagram of the resting (1), of the active muscle (2), and the curve of active force development (3). Essentially redrawn after an experimental curve of Ernst (1963) taken from a frog sartorius muscle. The abscissa has been transformed into a sarcomere length scale (S) assuming that the equilibrium length S_0 corresponds to 2.1 μ and the end of the active force development S_m to 3.6 μ (Gordon et al., 1964)

It is unnecessary to say that all our assumptions represent oversimplifications which we have made in order to be able to perform a comparatively simple calculation.

We have performed calculations by assuming effective forces to act only between neighbouring myosin and actin filaments. (The forces acting between myosin-myosin and actin-actin filaments, respectively, do not give any resultant force in the longitudinal direction.)

Method

Since according to electron microscopic investigations three myosin filaments are situated at the three vertices of an equilateral triangle, the actin filaments can be found at the centre of the triangle. Therefore if we take strictly straight lines for the shape of the filaments, the distance between a myosin and actin

filament will be $d/\sqrt{3}$ (see Fig. 2). We can try to take the force acting between an elementary length dx_2 of the myosin filament at the point x_2 and an elementary length dx_1 of the actin filament at the point x_1 as

$$p = \frac{A}{\rho^a} + \frac{B}{\rho^b}. \tag{2}$$

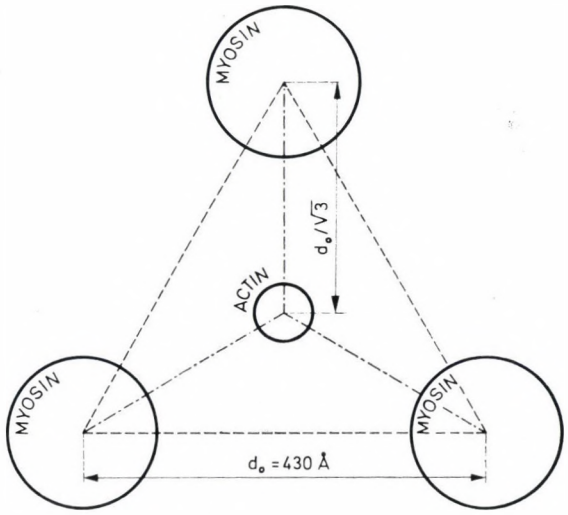

Fig. 2. The geometrical arrangement of three myosin filaments and one actin filament. The distance $d_0 = 430$ Å between two myosin filaments is taken by $S_0 = 2.1$ μ (Huxley, 1969)

Here A and B are constants, ρ is the distance between dx_1 and dx_2 (see Fig. 3) and a and b are integer number exponents ($b > a$). The component of p in the longitudinal direction x is

$$p' = p \cos \alpha = p \frac{x_2 - x_1}{\rho}. \tag{3}$$

Integrating p' over x_1 and x_2 we obtain the resulting force acting in the direction x:

$$P(S) = 3 \int_0^{S_0/2} dx_2 \int_{(S-S_0)/2}^{S/2} dx_1 \left(\frac{A}{\rho^{a+1}} + \frac{B}{\rho^{b+1}} \right) (x_2 - x_1). \tag{4}$$

Here S_0 is the equilibrium length of the sarcomere and S is its actual length due to an external stretching force. The factor 3 in eq. (4) is due to the fact that one actin filament is surrounded with three myosin filaments (Hanson, Huxley, 1955, see Fig. 3) and A and B are constants.

Let us denote the experimental length-tension curve shown in Fig. 1 by $R(S)$. Then we can write as the criterion of the best theoretical curve $P(S)$ to fit the curve $R(S)$ using the principle of least squares:

$$I(a, b) = \int_{S_0}^{S_m} [P(S) - R(S)]^2 \mathrm{d}S = \min. \tag{5}$$

(5) provides for the determination of the constants A and B the conditions

$$\frac{\partial I(a, b)}{\partial A} = \frac{\partial I(a, b)}{\partial B} = 0 . \tag{6}$$

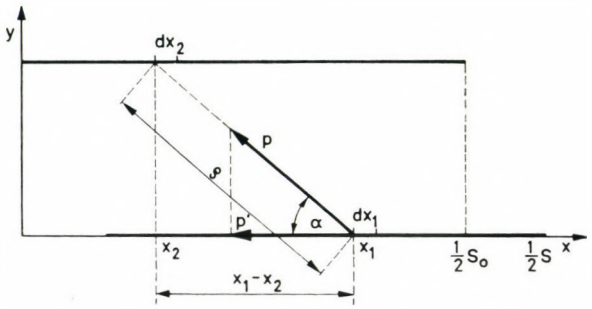

Fig. 3. The definition of quantities occurring in Equations (2) and (3). $\mathrm{d}x_2$ is situated on the myosin and $\mathrm{d}x_1$ on the actin filament, respectively

Since the value of the integral (5) is depending on the integers a and b, for each pair of a and b the constants A and B should be determined from (6) separately.

From equation (6) we obtain for the constants A and B, by given values of a and b, after a straightforward derivation the expressions

$$A_{ab} = \frac{V_a Q_b - V_b T_{ab}}{Q_a Q_b - (T_{ab})^2} ; \quad B_{ab} = \frac{V_b Q_a - V_a T_{ab}}{Q_a Q_b - (T_{ab})^2} , \tag{7}$$

where

$$V_r = \int_{S_0}^{S_m} R(S) I_r(S) \mathrm{d}S \quad (r = a, b) , \tag{8}$$

$$Q_r = \int_{S_0}^{S_m} I_r(S)^2 \mathrm{d}S \quad (r = a, b) , \tag{9}$$

$$T_{ab} = \int_{S_0}^{S_m} I_a(S) I_b(S) \mathrm{d}S . \tag{10}$$

Further

$$I_r(S) = F_r^0(S) - 2F_r^1(S) + F_r^2(S) \quad (r = a, b) , \tag{11}$$

where

$$F_1^i(S) = \frac{3}{4}\sqrt{(\tilde{K}/S)}\ [2u_i - u_i \ln (u_i^2 + 1) - 2 \operatorname{arctg} u_i], \quad (r = 1) \quad (12/a)$$

$$F_2^i(S) = 3 \ln (\sqrt{u_i^2 + 1} + u_i), \quad (r = 2), \quad (12/b)$$

$$F_3^i(S) = \frac{3}{\sqrt{\tilde{K}/S}} \operatorname{arctg} u_i, \quad (r = 3), \quad (12/c)$$

$$F_r^i(S) = \begin{cases} \dfrac{3}{4n^2 - 1}\left(\dfrac{4S}{\tilde{K}}\right)^n \dfrac{u_i}{[u_i^2 + 1]^{\frac{2n-1}{2}}} \displaystyle\sum_{k=0}^{n-1} C_k^n (u_i^2 + 1)^k \\ \qquad (r = 2n + 2 \text{ and } n \geqq 1), \qquad (12/d) \\ \dfrac{6}{2n + 1} \dfrac{4^n u_i}{[\tilde{K}/S]^{\frac{2n+1}{2}} [u_i^2 + 1]^n} \displaystyle\sum_{k=0}^{n-1} \frac{(u_i^2 + 1)^k}{(2n - 2k - 1) C_{k+2}^{n+2}} + \\ + \left(\dfrac{4S}{\tilde{K}}\right)^n \dfrac{F_3^i(S)}{(n + 1) C_n^{n+1}} \quad (r = 2n + 3 \text{ and } n \geqq 1) \quad (12/e) \end{cases}$$

In all these equations $i = 0$, 1 or 2

$$\tilde{K} = \frac{4}{3} K = \frac{4}{3} \cdot \frac{S_0 d_0^2 \sqrt{3}}{4}, \qquad u_i = \frac{S - iS_0}{\sqrt{\tilde{K}/S}},$$

$$C_k^n = \frac{(2n - 2)(2n - 4) \ldots (2n - 2k)}{(2n - 3)(2n - 5) \ldots (2n - 2k - 1)}.$$

After calculating the constants A_{ab} and B_{ab} for given values of a and b with the aid of equations (5) to (10) we can calculate the theoretical curve $P_{ab}(S)$ with the aid of (4). (The results of this integration had to be used also in obtaining the expressions (7) of the constants A_{ab} and B_{ab}.) One obtains in this way

$$P_{ab}(S) = A_{ab} I_a(S) + B_{ab} I_b(S). \quad (13)$$

Substituting (13) with definitions (7) and (11) into (5) one can calculate numerically for different values of a and b the integral $I(a, b)$ which measures the error of $P(S)$ as compared to $R(S)$.

In the course of this integration the experimental curve $R(S)$ was divided into 16 parts between $S_0 = 2.1\mu$ and $S_m = 3.6\mu$. The division points have been connected by straight lines.

The numerical integrations (5), (6), (9) and (10) have been performed with the aid of Simpson's rule. The calculations have been done for the three series $a = 6$, $b = 7(1)18$; $a = 7$, $b = 8(1)18$; $a = 8$, $b = 9(1)18$.

Results and discussion

The results of the calculation are shown in Table 1 for the above-mentioned values of a and b. We can see from the values $I(a, b)$ given in the second column that the best fit was obtained for $a = 7$ and $b = 15$, although the values $I(6, 15)$ and $I(8, 15)$ are almost as good as $I(7, 15)$. At the same time the best value for exponent b of the repulsive force is more sharply defined. Finally, in the last two columns we have given the constants $A(a, b)$ and $B(a, b)$. In Fig. 4 we show the best theoretical curve ($a = 7$), $b = 15$ and the experimental one.

For the exponent a of the attractive force the obtained optimal value of 7 is the value usually occurring in the case of van der Waals forces. The found optimal value of the exponent of the repulsive force ($b = 15$) is somewhat large. (By some

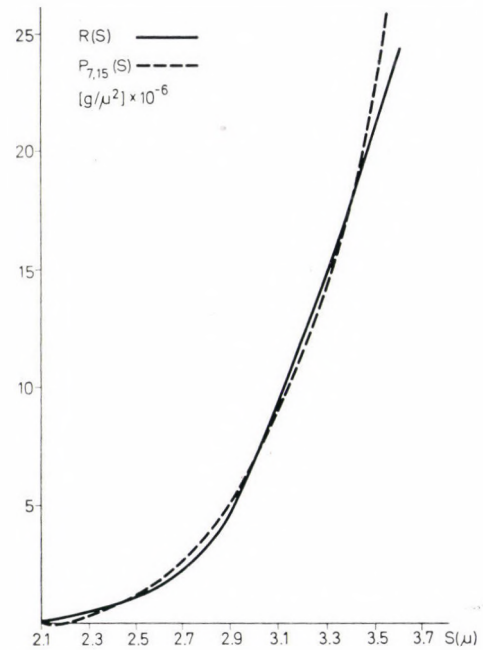

Fig. 4. The theoretical length tension curve $P(S)$ (broken line) and the experimental length-tension curve $R(S)$ (continuous line) at $a = 7$ and $b = 15$

ionic crystals b values as large as 11 to 12 have been found (Seitz, 1940) and the usual b value for repulsion between neutral molecules is 13.) This is probably due to the rough approximations applied in the course of the calculation.

According to the sliding model in the resting muscle there is only a very weak interaction between myosin and actin filaments (Huxley, 1960, 1969 and

Table 1

Error integral values and the constants $A(a, b)$ and $B(a, b)$ of the attractive and repulsive forces, respectively, at different integers a and b

b	$I(a, b)$ $[g^2/\mu^3] \times 10^{-12}$	$A(a, b)$ $[g\mu^{a-4}] \times 10^{-6}$	$B(a, b)$ $[g\mu^{b-4}] \times 10^{-6}$
		$a = 6$	
7	2.81	1.92×10^{-6}	-4.23×10^{-8}
8	1.31	1.08×10^{-6}	-3.30×10^{-10}
9	1.34	6.08×10^{-7}	-4.73×10^{-12}
10	0.83	4.42×10^{-7}	-3.67×10^{-14}
11	1.03	2.32×10^{-7}	-6.58×10^{-16}
12	0.54	2.31×10^{-7}	-5.00×10^{-18}
13	0.40	1.58×10^{-7}	-1.23×10^{-19}
14	0.45	1.30×10^{-7}	-7.33×10^{-22}
15	0.30	7.83×10^{-8}	-2.10×10^{-23}
16	0.50	5.36×10^{-8}	-1.05×10^{-25}
17	0.38	3.06×10^{-8}	-3.51×10^{-27}
18	0.69	1.66×10^{-8}	-1.59×10^{-29}
		$a = 7$	
8	2.68	3.92×10^{-8}	-5.64×10^{-10}
9	0.94	1.86×10^{-8}	-6.64×10^{-12}
10	0.90	1.17×10^{-8}	-4.53×10^{-14}
11	0.55	7.67×10^{-9}	-8.70×10^{-15}
12	0.58	5.53×10^{-9}	-5.55×10^{-18}
13	0.34	3.53×10^{-9}	-1.30×10^{-19}
14	0.49	2.58×10^{-9}	-7.42×10^{-22}
15	0.29	1.77×10^{-9}	-2.20×10^{-23}
16	0.54	1.36×10^{-9}	-1.10×10^{-25}
17	0.39	6.56×10^{-10}	-3.55×10^{-27}
18	0.70	2.60×10^{-10}	-1.58×10^{-29}
		$a = 8$	
9	11.21	5.07×10^{-11}	-2.62×10^{-12}
10	0.60	2.30×10^{-10}	-6.22×10^{-14}
11	0.76	1.27×10^{-10}	-1.02×10^{-15}
12	0.45	9.03×10^{-11}	-6.37×10^{-18}
13	0.40	5.17×10^{-11}	-1.39×10^{-19}
14	0.47	3.72×10^{-11}	-7.76×10^{-22}
15	0.31	2.59×10^{-11}	-2.20×10^{-23}
16	0.53	2.00×10^{-11}	-1.14×10^{-25}
17	0.40	9.08×10^{-12}	-3.59×10^{-27}
18	0.71	3.36×10^{-12}	-1.58×10^{-29}

personal communication). This assumption is based on the observations and considerations concerning the active force development (Gordon et al., 1963, 1966a, 1966b). According to their results the active force developed by the muscle is proportional to the number of the possible interaction sites (i.e., to the number of overlapped cross-bridges) at a given length. It is to be noted that on the flight muscle of the bee (i.e., on a specimen of quite different sarcomere and filament lengths) we arrived at an essentially similar result (Belágyi, Garamvölgyi, 1968; Garamvölgyi, 1971). It follows from this that the active force development is independent of the lateral spacing and that the active force will not be influenced by the resting elasticity.

It is evident that the resting elasticity cannot hinder the actively shortening muscle because the elastic forces necessarily point towards the direction of shortening of a stretched muscle. This is the reason why elastic forces can by no means weaken the active force development. On the other hand, the tension of the active muscle within the range extending from the slack length up to the no-overlap point is not independent of the resting elasticity, since at all lengths within this range it is equal to the sum of the resting tension plus active force development. The active force development is in fact dependent on the resting tension due to its definition as the difference of the length-tension curves of the active and resting muscles, respectively. Nevertheless, the independence of the active force development on the lateral spacing is of a great functional importance (Huxley, 1969). This will be discussed in the next paper of this series.

It is true that in the environment of the resting length where the active force development has its highest values, the resting tension is very low. Nevertheless, it is sufficient to make the muscle shorten, when it is to be excised. This means that in the organism the muscles always stand under a certain mechanical tension. This very important fact may be helpful in theoretical considerations concerning the mechanical action of the cross-bridges themselves, but our considerations do not interfere in any respect with any experimental results serving as basis of the sliding model.

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Build-up Measurements in Air Cavities Irradiated with 6 MV or 42 MV X-rays

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The electronic build-up effect and its influence on the absorbed dose around an air-tissue interface was investigated by 10^{-2} mm TLD discs in a polystyrene phantom. The air cavity was situated at different depths in the phantom and the build-up curve in the cavity wall was determined. The results suggest that the reduction of the absorbed dose at the cavity surface must be taken into consideration in radiotherapy, at least when the cavity size is of the order of several cms.

Introduction

In all cases when high energy photons are passing through the interphase between materials, e.g. from air to tissue, a lack of electronic equilibrium can be observed in the region near to the surface. By this process the energy dissipated by forward scattered electrons constitutes a dose maximum where the electronic equilibrium has been established. This effect leads to a reduction of the absorbed dose in the material around the interphase. In layers beyond an air space the phenomenon of electronic build-up prevails. This problem has been investigated in the high-energy range by several authors (Dutreix et al., 1962; Bernard, 1964; Dutreix, Bernard, 1966; Guillie et al., 1972).

When an air-filled cavity is in a medium the main parameters modifying the character of the build-up curve are the shape, size and depth of the cavity, as well as the beam size and the radiation energy. A number of investigations have been performed using ^{60}Co radiation and other high energy photons by ionization chamber (Burlin, 1957; Epp et al., 1958) and also by TLD (Carlsson et al., 1969; Scrimger, 1972).

The present investigation on the dose distribution and on the build-up effect in particular, was performed in order to verify previous measurements on ^{60}Co and to extend the investigation to other cavities as well as other photon energies. The method and the results were as follows.

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Methods

A Clinac 6 and a Siemens betatron were used as radiation sources exposing the phantom with 6 MV and 42 MV X-rays. The dose measurements were carried out with LiF Teflon discs approximately 1.3×10^{-2} mm in thickness (read out by Con-Rad 5100 A TLD reader). 40 LiF discs were individually calibrated by irradiation with the same doses of 6 MV and 42 MV X-rays. The calibration was performed at 90 cm or 100 cm SSD, 10×10 cm^2 beam size under condition of electronic equilibrium. An individual correction factor was determined for each dosimeter calculated from five measurements. The standard deviation of readings was less than 1 per cent for 10 discs, between 1 and 2 per cent for 22 discs and more than 2 per cent for 8 dosimeters.

A 10.8 cm thick polystyrene phantom with an air gap was set up at 90 cm and 100 cm SSD, resp., and irradiated with approximately 300 rads. The irradiation was made by 4×16 cm^2 beam size at the Clinac 6 and by 4×4 cm^2 at the betatron. A 3.9 cm thick polystyrene layer was used in the front side of the air cavity. The irradiation geometry is shown in Fig. 1. In this arrangement a number of discs were set onto the cavity surface and in triplicate in different depths varied between 0.1 and 4.5 cm. The zone of electronic build-up and the dose distribution could thus be determined from at least three readings for each measuring point.

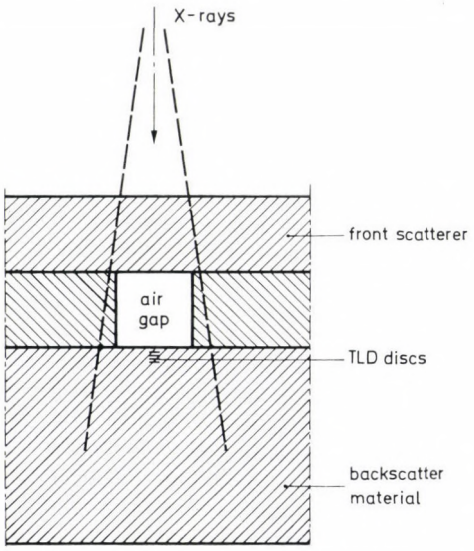

Fig. 1. Irradiation geometry for measuring the electronic build-up

Results and conclusions

Relative depth doses in the centre of the beam were measured at 6 MV in a parallelepiped cavity open at both ends, 3 cm in width and 3.9 cm in depth. At 42 MV an open-ended cavity of 3 cm width and 6.9 cm depth was used. The build-up curves obtained at these parameters are shown in Fig. 2. The maxima of the absorbed dose were found at 0.3 – 0.4 cm and 3 – 4 cm depths, resp., for the two energies (Peterson et al., 1972).

In the case of a photon beam passing through an air-tissue interface the absorbed dose at the inner surface of the gap will be less than its value at electronic equilibrium. Consequently, the decrease of the absorbed dose in tissues being in vicinity of the gap should be considered. This means that the build-up effect must be taken into account in therapy with high energy radiation when an air cavity is situated in an irradiated volume. In the present experiments the relative dose distribution was measured by using LiF Teflon discs which have a small mass, thereby not influencing the dose values.

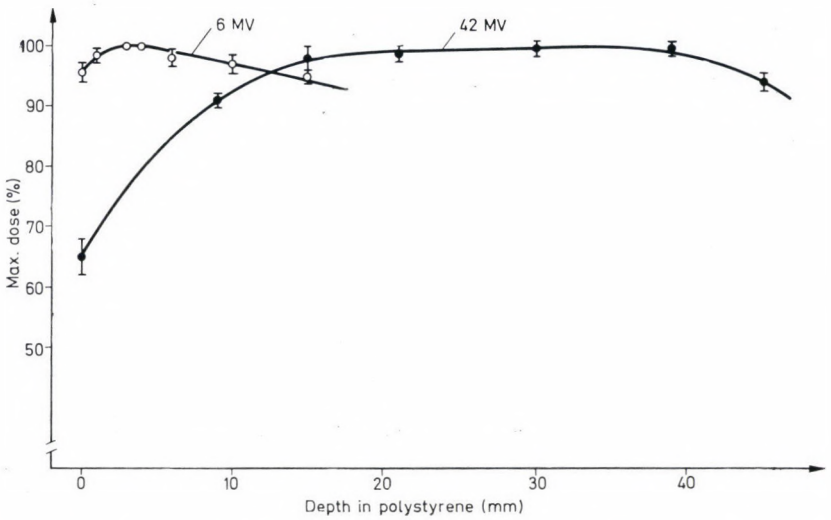

Fig. 2. Build-up curves in polystyrene phantom. o——o 6 MV, 4×16 cm² beam size; ●——● 42 MV, 4×4 cm² beam size

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Thick Filaments in Guinea Pig Vas Deferens Smooth Muscle

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(Received October 4, 1972)

In connection with the discussion concerning the contractile mechanism of vertebrate smooth muscle two different kinds of filaments were previously found in intestinal and blood vessel muscles. In the present paper the authors demonstrate the presence of thick filaments in an additional kind of vertebrate smooth muscle, i.e. in urogenital smooth muscle. In this relation the effect of tension on the preservation of smooth muscle myosin filaments is discussed.

Introduction

In a previous paper (Garamvölgyi et al., 1971) we described the regular occurrence of thick filaments in a guinea pig intestinal muscle of highly advantageous experimental properties (longitudinal layer strips from the ileum), provided that the strips were fixed at a constant length higher than the slack length of the muscle. This means that we found thick filaments in muscles fixed in a slack state where usually no thick filaments could be identified. We observed the same effect on atropine-relaxed muscles, too, therefore we denied the role of stretch activation in the formation of the thick filaments (Kelly, Rice, 1969). We claimed the thick filaments to be regular constituents of vertebrate smooth muscles, too. We assumed that, in the case of stretch, polymer crystallization (e.g. Ernst, 1963) might prevent the destruction of myosin filaments caused by the electron microscopic procedure. In another paper (Garamvölgyi, 1971) we demonstrated the artificial annihilation of myosin filaments also in a striated muscle (bee wing muscle, glycerinized in a stretched state and released before fixation) as a consequence of fixation, i.e. as an obvious artefact.

Recently Somlyó and co-workers (1971) claimed to have found both kinds of filaments in mesenteric vein smooth muscle, even in the unstretched state. The above authors as well as Cooke and Fay (1972) (the latters working on *Taenia coli*) do not agree with our earlier statement made on the importance of mechanical tension in the preservation of myosin filaments.

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Materials and methods

Vasa deferentia of the guinea pig were prepared according to a method used for pharmacological purposes (Knoll et al., 1972). They were quickly liberated from elements of the mesenterium and immediately dropped into 2.5 per cent glutaraldehyde. After a prefixation of 3 hours the muscles were postfixed in 1 per cent osmium tetroxyde for one more hour. The specimens were embedded in Durcupan Fluka ACM araldite, sectioned with an LKB "Ultrotome III" ultramicrotome, contrasted by uranyl acetate–lead citrate and studied in a WF-SEM-3 electron microscope.

Results and discussion

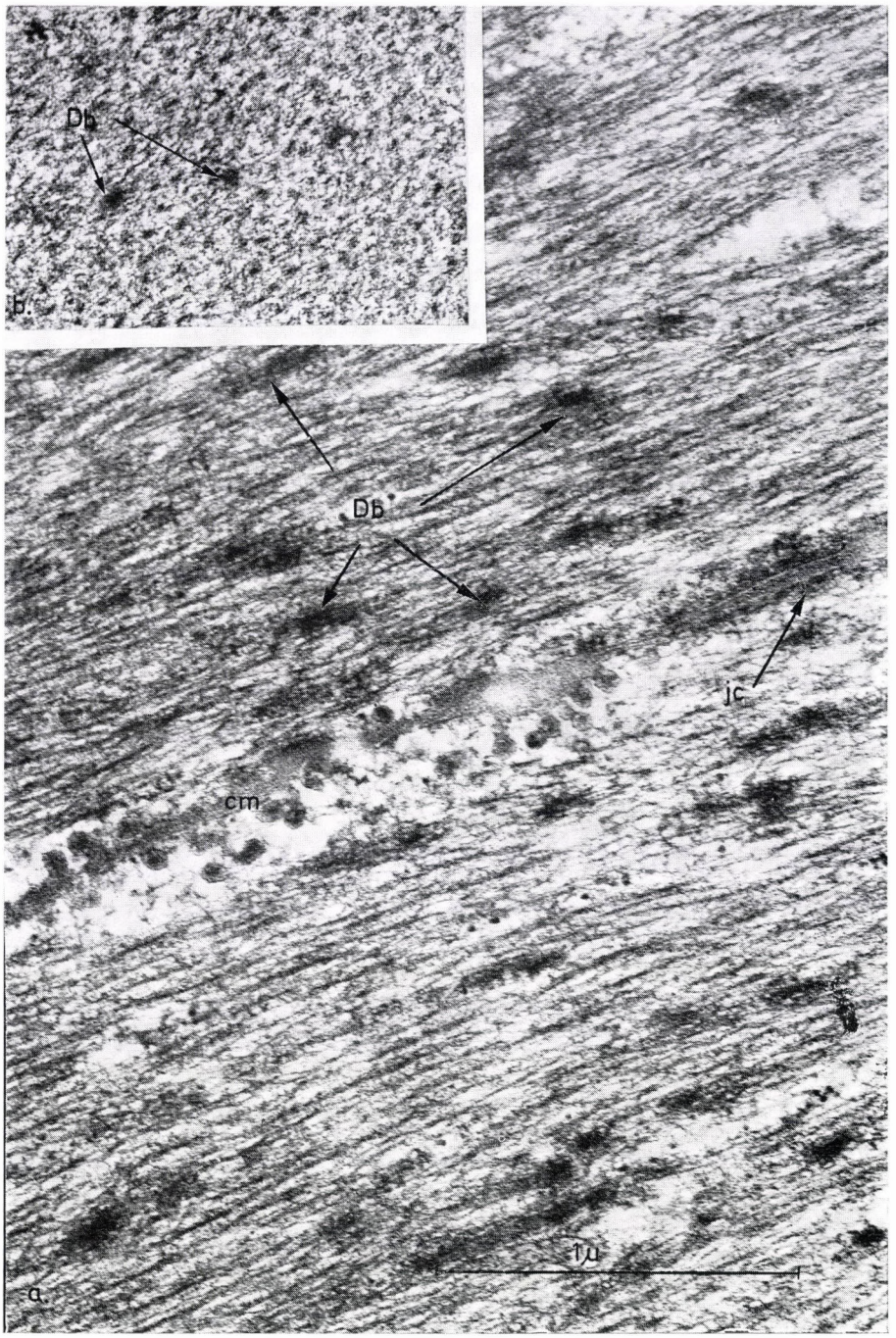
The cells of the vas deferens muscle are by no means parallel, there are bundles of a few cells running in different directions. It is therefore relatively difficult to find cells of exactly longitudinal or transverse orientation. Contrary to intestinal smooth muscles, the extracellular space is very small, the membranes of adjacent cells closely fit together. There is a large number of junctional complexes consisting of two exactly parallel cell membranes with dense material between them as well as on both inner surfaces of the cell membranes. They are larger than the typical desmosomes and the amount of the dense material seems relatively low. At any rate, they seem to represent firm mechanical connections between the individual cells being most probably able to prevent their relative displacement (Fig. 1a).

We regularly found both kinds of filaments independently of the orientation of the cells. In nearly perfect transverse sections (Fig. 1b) the cross-section of the thick filaments is rather circular and no indications of a ribbon-like association of myosin was observed (Lowy, Small, 1970). Apart from the thick and thin filaments there is a large number of dense bodies in all cells.

The muscles in our specimens were not deliberately stretched. Notwithstanding, thick filaments were present virtually without exception. Earlier, Somlyó et al. (1971) reported on the same result obtained on mesenteric vein muscle fixed without any previous stretch. Neither of these both types of muscle consist, however, of parallelly arranged cells and mechanical tension exerted by the firmly attached neighbouring cells on each other can hardly be excluded, particularly when the cells contract under the influence of the fixative. Neither the observation of Somlyó and co-workers (1971), nor our present observation contradict our original statement on the effect of tension on the state of smooth muscle myosin (Garamvölgyi et al., 1971).

Fig. 1a. Longitudinal section of the vas deferens smooth muscle of the guinea pig. Thick filaments are clearly recognizable. Db: dense bodies; cm: cell membranes; jc: junctional complex

Fig. 1b. Transverse section from the same muscle as shown in Fig. 1a. Same magnification


Db
b.
Db
ic
cm
1μ
a.

Cooke and Fay (1972) used *Taenia coli* muscle, but they were not able to correlate the presence of thick filaments with the necessity of a stretch beyond the excised length of muscles. It turns out, however, from the Materials and Methods that they also fixed their specimens in their isometric state, though the resting tension was in fact relatively low. They do not mention any totally slack controls. In our experiments, too (Garamvölgyi et al., 1971), performed on longitudinal strips of the ileum, very low degrees of stretch were sufficient to make thick filaments consistently observable. In contrary, they were missing from many cells when the muscles were fixed just at the excised length, avoiding any stretch.

At any rate, it seems now well established by the numerous observations of different authors made on different specimens that all vertebrate smooth muscles represent two-filament systems.

We are indebted to Dr. P. Illés and to Mrs. Helen Garamvölgyi for the preparation of the specimens.

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Book Reviews

Physical Problems in Biological Systems: Lecture notes of the Les Houches Summer School, 1969, under the direction of C. De Witt and J. Matricon. Gordon and Breach Science Publishers Ltd. London 1970. 452 pages

The Summer School organized by the International Atomic Energy Agency and by NATO in 1969 set two aims: to call the attention of physicists to biological — particularly to molecular biological — problems and to arouse the interest of biologists in physical methods.

In his introductory lecture H. Chanterence elucidated the main principles and basic concepts of molecular biology, thus removing the greatest hobgoblin which deters physicists from working on biological problems. In this introductory lecture clear definitions are given of almost ail phenomena, such as enzymatic reactions, translations of genetic information, immunreaction, etc., which are unknown to physicists and thereby the under standing of further lectures is made possible.

Some of the papers have earlier been published of these only the list of references is presented. The work on the X-ray diffraction of proteins is similarly reported; since this covers more than a simple paper and would also need a long introduction, a detailed list of references is provided for those interested.

Two papers on lambda-bacteriophages, three papers on membranes and one about haemoglobin are reported in full length. Three methodological papers on the application of nuclear magnetic resonance, electron paramagnetic resonance and optical properties of biopolymers, can also be read. Finally, two short theoretical papers can be found in the book: "Intermolecular forces and protein structure" and "Phase transitions in one-dimensional systems".

Contents of the lectures, according to the original papers, are reported in English or French.

I. SIMON

Alfred Benson Symposium IV. Oxygen Affinity of Hemoglobin and Red Cell Acid Base Status Ed. M. Rorth and P. Astrup, Munksgaard, Copenhagen, 1972.

In May 1971 the IVth Alfred Benson Symposium was held with the aim to gather the experimental and clinical experiences on the oxygen carrier capacity of blood, and on the regulatory mechanisms governing it. The lectures and main discussions at the Symposium are compiled in this book, excellently covering some of the major aspects of the relation between red cell structure and function. The 52 contributors of the Symposium presented their most recent results in the molecular biology, biophysics, physiology and clinics of the oxygen transport properties of blood. The most prominent representatives of the interdisciplinary research work on oxygen supply could be found among the participants of the Symposium.

The lectures comprise studies on hemoglobin structure, red cell metabolism and permeability. They are arranged in 9 chapters, according to their special topics.

The molecular biology of hemoglobin has been elucidated to a remarkable depth due mainly to stereochemical studies by means of X-ray crystallography. The exact knowledge

of the molecular structure permits one to visualize the functional properties of hemoglobin, since reversible oxygen binding by hemoglobin is highly dependent on the tertiary and quaternary structure of the molecule and is influenced by red cell metabolites, too.

The first chapter gives an insight into the interactions between hemoglobin and oxygen on the structural level, together with the factors influencing them. In this frame the effects of certain anions, cations and organic phosphates, as well as the modified oxygen dissociation curves of hemoglobin, are described. The hemoglobin–oxygen interaction is highly pH dependent. The molecular interpretation of this phenomenon, called Bohr effect, is also treated in this section. The mathematical analyses of oxygen dissociation curves and in general, of the hemoglobin–ligand reactions, complete the discussion of this problem.

The oxygen affinity of hemoglobin is influenced by the metabolic activity of the red cell and by pCO_2 as well. Therefore, several papers deal with the interactions of hemoglobin and carbon dioxide. Here the carbamate equilibrium between hemoglobin and CO_2, the specific effect of CO_2 on oxygen affinity of hemoglobin and the effect of organic phosphates on CO_2 binding and transport, are the main topics. The discussion of these interactions requires a description of diffusional problems of O_2 and CO_2, and the structure and function of carbonic anhydrase.

Another section is devoted to the ion permeability of the erythrocyte membrane, especially with regard to H^+, OH^- and Cl^- ions. A short survey on membrane potential and resistance is also given.

The regulation of red cell metabolism by 2.3 DPG level is discussed in detail. The role of biochemical, physiological and genetic factors in red cell metabolism and 2.3 DPG level, as well as the regulation and adaptation of 2.3 DPG metabolism during hypoxia constitute a sizeable part of the book.

The last two chapters summarize the physiological aspects of the oxygen affinity of hemoglobin. In this part clinical implications are discussed, with special reference to the conditions during pregnancy, foetal and neonatal life.

Highly interesting considerations are given to two examples of increased oxygen affinity, viz. the acute effect in the transfusion of high affinity (stored, 2.3 DPG depleted) blood tissue oxygenation, and the chronic one, i.e. the role of increased erythrocyte oxygen affinity in the pathogenesis of arteriosclerosis.

In conclusion the book treats the biological regulation of the oxygen affinity of hemoglobin. The discussion of adaptability and other physiological factors influencing the interaction between hemoglobin and oxygen completes the many-sided picture of the oxygen supply of the organism.

The editors arranged the papers with much logic and compiled the book with great expertness. The lectures with a great number of figures, tables and references, are supplemented by the main comments and contributions, and so provide both a relevant and an up-to-date picture of the subject being in the focus of interest nowadays.

Judith SZELÉNYI

Antigen–Antibody Reactions. Contributions to the IVth Symposium on Immunology of the "Gesellschaft für Allergie und Immunitätsforschung der DDR", Leipzig 3–5 Dezember, 1970. Edited by H. Amborius, K. Malberg, H. Schäffer. VEB Gustav Fischer Verlag, Jena 1971.

The reaction between antigen and antibody is the central phenomenon of immune response. Specific binding of antibodies to antigens triggers the non-specific effector mechanisms, like complement activation and different kinds of hypersensitivity reactions. Convincing evidence has been accumulated that even the cellular events leading to the active production of specific antibodies are elicited by an antigen–antibody reaction: the interaction between cell-bound receptors, membrane-bound antibodies, and antigens. It is easy to imagine therefore that under the title of this symposium a broad variety of immunological topics could be gathered. Indeed the presentations cover highly divergent aspects of basic and applied immunology. It is impossible to report all fifty papers, therefore this review will remain a rather subjective selection.

In the first part, entitled "Basic principles of antigen–antibody reactions" a number of papers are focused on the problems of the affinity of antibodies and the phylogenesis of immune response. H. Fiebig (Leipzig) presented exact thermodynamic data on the affinities of antibodies against the dinitrophenyl (DNP) hapten. The data show a great increase of affinity during the course of immunization. The affinity has been measured against a series of different DNP-haptens and a narrow more restricted specificity for the antibodies was demonstrated. The author interprets this result "as a selection to antibodies with a better fit of the combining site to the lysine part of the hapten". Two related papers, read by R. Richter and E. M. Frenzel (both from Leipzig) report studies on immune reactions of lower vertebrates. Both authors conclude that antibodies of lower vertebrates must have relatively large combining sites with a broad specificity, resembling a type of antibody appearing in the early immune sera of mammals. K. Hummel (Freiburg i. Br.) gave a review of the physico-chemical aspects of antigen–antibody reactions. Doing this he always emphasized the practical consequences which can be drawn from determining thermodynamical and kinetic data. Among others he pointed out that the degree of adaptation of antibody to the antigen is also important in the regulation of antibody production through a feed-back mechanism.

Antibodies of higher affinity are more effective in the feed-back regulation than lower affinity antibodies. W. Ptak (Cracow) presented experimental results indicating that antibody-mediated suppression of immune response can take place at the level of handling the antigen by macrophages. T. Szilágyi et al. reported experiments on various antibody-mediated hypersensitivity reactions elicited in *Rana esculenta*. They have found immediate type hypersensitivity, too. An interesting finding was that in frogs histamine did not produce the smooth muscle effect well known in warm-blooded animals.

Affinity labelling is the most efficient tool for locating the antibody-combining site on the immunoglobulin molecule. This principle has been applied by F. Franek (Prague) to the study of pig anti-DNP antibodies. The labelling of tyrosine 33 of the lambda chain suggests that one of the hypervariable regions is involved in the active site. J. Novotny and F. Franek (Prague) concluded, on the basis of comparing sequences, that the N-terminal 23 amino acid residues of immuno-globulin light chains do not take part in the formation of the antibody active site.

The second part of the symposium was devoted to methodology. A number of papers report the experiences obtained with highly sensitive modern techniques of antigen or antibody detection.

E. Rejnek (Prague) pointed out that the technique of radio-immunodiffusion is not only a suitable method for the quantitation of protein antigens at the nanogram level, but it can also be used to detect the primary interaction between antibody and antigen. I. Riha discussed the problems of the widely used methods of indirect haemagglutination and haemolysis. As. Toshkow and L. Shirova (Sofia) exploited the unusual behaviour of erythrocytes treated with the Vi-polysaccharide described by Ceppellini and Landi in 1963. Erythrocytes carrying the Rh_0 (D) antigen after such a treatment are not agglutinated by complete anti-D antibodies, although antibodies are bound to the cells. The sensitized erythrocytes were used to detect anti-immunoglobulin factors in sera of rheumatoid patients. Unfortunately, however, no attempt to determine the specificity of these factors was reported. The use of antibody coupled to ferritin, peroxidase or a ferrocenyl derivative for the ultrastructural localization of cell membrane antigens by electron-microscopy was discussed by B. Micheel et al. (Berlin). They demonstrated the use of combined techniques for the simultaneous detection of two different antigens.

The papers in the third part cover various topics of clinical and experimental immunopathology. Four papers were read on autoimmune phenomena observed in New Zealand Black mice. V. I. Levenson (Moscow) studied the interaction of early or late antibodies produced against the Vi-antigen of *S. typhi* with macrophages. He found cytophilic activity of the early, IgM type, antibodies only if these had previously been combined with the antigen. As a conclusion he outlined the challenging concept of the secondary, antigen-induced, cytophily of certain antibodies.

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Isolation and Properties of Feedback-insensitive DAHP Synthase (phe) Mutants of *E. coli* K 12

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(Received June 12, 1972)

Feedback-insensitive mutants of *E. coli* K12 were isolated with the aid of a false feedback inhibitor beta-2-thienylalanine. The phenylalanine-excreting strains were selected and it was found by genetic mapping that the mutation affected the aro-G gene, the structural gene for DAHP synthase (phe), E.C. 4.1.2.15.

The feedback-insensitive DAHP synthases were partially purified from the mutants and studied with respect to repression behaviour, phenylalanine binding, inhibition and stability of enzyme activity at elevated temperatures.

We could not detect mutants in which feedback insensitivity and repression behaviour altered simultaneously.

Phenylalanine has several effects on the structure of the enzyme: binding of this amino acid can inhibit, stabilize and activate the enzyme.

There is no correlation between phenylalanine binding and inhibition of enzyme activity, by the amino acid.

Introduction

In *E. coli* the first step of the aromatic amino acid biosynthetic pathway is controlled by the effect of the three aromatic amino acids on the levels and activities of the three cognate DAHP synthases (E.C. 4.1.2.15) (Smith et al., 1962; Doy, Brown, 1965). These enzymes, catalyze the conversion of erythrose-4-phosphate and phosphoenolpyruvate to DAHP.

Isoenzymes 1a and 1b are sensitive to allosteric inhibition by phenylalanine and tyrosine, respectively. The activity of isoenzyme 1c is not inhibited by any of the aromatic end products. The phenylalanine sensitive isoenzyme 1a is the most important of DAHP synthases of wild type *E. coli* cells grown on minimal medium, as it gives about 80—90% of the total DAHP synthase activity (Wallace, Pittard, 1967).

Suggestive evidence indicates that the enzymological, biochemical and genetic regulations are not independent of each other and in some cases the allosteric enzyme of a biosynthetic pathway might play a role in the repression

Abbreviations: DAHP, 2-oxo-3-deoxy-D-*arabino*-heptulestonate-7-phosphate; PEP, phosphoenolpyruvate; E4P, erythrose-4-phosphate; TA, thienylalanine; gal, galactose operon; thi, thiamine.

process of the enzymes of that pathway (Sommerville, Yanofsky, 1965; Berberich, Gots, 1965; Kovach et al., 1969).

We have shown a specific interaction between phenylalanyl-tRNA and purified DAHP synthase (phe) (Duda et al., 1968). We intend to study the role of this complex in regulation by the aid of mutants in which the alteration of the enzyme is accompanied by anomalous repression behaviour and tRNA binding.

For this reason we tried to isolate and study mutants of the phenylalanine-sensitive isoenzyme with altered regulatory properties and/or repression behaviour.

The present report describes the isolation and properties of feedback-insensitive mutants.

Materials and methods

Chemicals

Thiobarbituric acid was obtained from Fluka, Buchs. Beta-2-thienylalanine was a product of Sigma Chem. Comp., St. Louis; E4P was prepared according to Ballou (1962); hydroxyapatite was prepared by the method of Levin (1962); all other chemicals were reagent grade preparations of Reanal, Budapest.

Organisms

The bacterial strains used in this work are all derivatives of *E. coli* K 12 lambda, (gal^-) isolated from wild type K 12 lambda on eosin–methylene-blue–galactose medium, according to Clowes and Hayes (1968) except for Hfr H, thi^- and K 10-3 phe^- straubism which were gifts of Dr G. Dénes.

Mating was carried out as described by Clowes and Hayes (1968). Excretor mutant strains were mated with Hfr H (thi^-, aro-G^+, gal^+) in a complete medium and spread on galactose-containing minimal plates after thorough washing with sterile minimal medium. Gal^+, thi^+ recombinants were tested for TA-resistance and phenylalanine excretion.

Isolation of regulatory mutants

Cells in the logarithmic phase were washed with minimal medium A (Davis, Mingioli, 1950) and spread on minimal plates containing 100 μg/ml TA. $10^8 - 10^9$ cells were spread on one plate, resistant clones arising by spontaneous mutation were isolated and recloned (about 6000 colonies).

Resistant colonies were tested for phenylalanine excretion on minimal plates containing K – 3 phe^- cells. Excretors were readily detected as colonies surrounded by halos of background growth of K 10 – 3 cells. The excretor strains were purified on other TA-containing plates.

Preparation and assay of DAHP synthase

For enzyme preparation cultures were grown on a rotatory shaker and centrifuged in the late logarithmic phase with $5000 \times g$ for 30 minutes. After washing with 0.15 M sodium chloride solution the cells were suspended in 0.2 M potassium phosphate buffer, pH 7, for the preparation of crude extracts or stored in the frozen state ($-15°$).

Cells were disrupted in an Aminco French pressure cell at 15 000 psi and centrifuged with $25\,000 \times g$ for 30 minutes. The supernatant was mixed with 0.82 volumes of cold ($-20°$) acetone, during a 5 minute period while the temperature was kept below $-10°$. After centrifugation at $-10°$ the supernatant was mixed another 0.41 volumes (of the original) of cold acetone in the same way, and the precipitate was centrifuged.

The precipitated proteins and potassium phosphate were dissolved in ice-cold distilled water, centrifuged again and the supernatant was dialyzed against 10 mM potassium phosphate buffer, pH 7.0.

Hydroxyapatite chromatography was carried out at 0° on columns of the same volume as that of the cell paste. Equilibration and washing were performed with 100 mM potassium phosphate buffer, pH 7.0, and the enzyme was eluted with 200 mM of the same buffer. This procedure yielded a DAHP synthase preparation purified about 100-fold relative to the crude extract. Some mutant enzymes showed altered behaviour during acetone fractionation and precipitated at lower concentrations of acetone.

The enzyme was assayed as described by Staub and Dénes (1969a) with the only modification that the reaction mixture was buffered by 50 mM potassium phosphate, pH 7.5, instead of tris. The inhibition of enzyme activity was measured in the presence of 1 mM L-phenylalanine or L-tyrosine. This concentration causes about 90% inhibition of the wild type DAHP synthases (phe) and (tyr).

Protein was measured according to Lowry et al. (1951).

Heat inactivation

The enzyme samples were inactivated in a 50° water bath in 50 mM potassium phosphate buffer (pH 7) without substrates, in a dilution which prevents renaturation (Staub, Dénes, 1969b; Duda, Sasvári, unpublished results). After heat treatment the samples were cooled to 0° for the same reason.

Results and discussions

TA is a potent phenylalanine analogue. It inhibits the DAHP synthase (phe) to nearly the same extent as phenylalanine does, it is activated by the phenylalanine-amino-acyl-tRNA synthetase and is incorporated into proteins, thereby killing the sensitive cells (Munier, Cohen, 1959; Conway et al., 1962; Ezekiel, 1965).

Theoretically, cells can grow on TA-containing minimal media (supplemented with the growth factors required by the strain used) in the cases of one (or more) of the following mutations.

1. Mutations resulting in the ability to destroy TA permease mutations (mutants cannot transport TA into the cells), and phenylalanyl-tRNA synthetase mutations (mutants cannot activate TA).

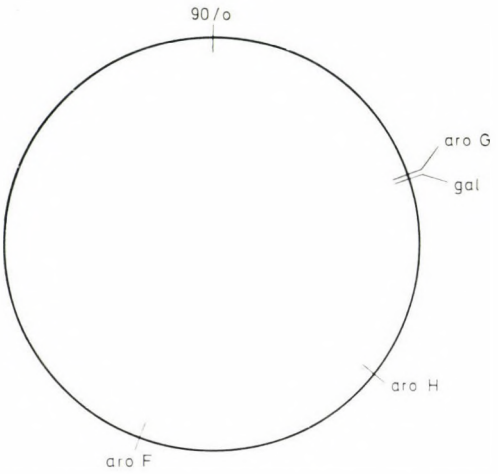


Fig. 1. Mapping of DAHP synthase structural genes on the *E. coli* chromosome. aro-F, aro-G and aro-H, structural genes for DAHP synthases (tyr), (phe) and (trp), respectively

This group of mutations causes TA-resistance without phenylalanine overproduction.

2. Mutations which lead to feedback insensitivity of DAHP synthase (phe) or (tyr) and probably constitutive mutations of one of the DAHP synthases.

This group of mutations causes TA-resistance and phenylalanine excretion.

We have been interested in mutants with feedback-insensitive DAHP synthase (phe), mutants with non-regulated levels of DAHP synthase (phe) and especially mutants in which a single mutation led to feedback-insensitivity and altered repression behaviour.

We selected the desired type of mutants by using the fact that the first group of mutants do not overproduce phenylalanine. The excretor strains were mapped and the aro-G mutants (closely linked to gal operon) were used.

Measuring the DAHP synthase activity in crude extracts of excretor strain in the presence and absence of 1 mM phenylalanine, we selected some 60 phenylalanine (and TA), feedback-insensitive strains. All the excretor, feedback-insensitive strains investigated showed 100% linkage between the gal operon and the site of the mutation.

None of the mutants isolated by this procedure exhibited anomalous repression behaviour. (The repressed enzyme levels were measured in dialyzed crude extract of cells grown in minimal medium supplemented with 1 mM phenylalanine. Because cells excrete phenylalanine we sedimented the cells from the culture every hour during growth and suspended them in sterile minimal media. In the supernatant the concentration of phenylalanine remained below 10^{-5} M, as determined by the aid of the K 10−3 strain.)

The derepression in the wild type strain is about 2−2.5-fold. In feedback-insensitive mutants it varies between 1.7−2.6-fold. (In the group of constitutive mutants − not discussed here − it is below 1.7 fold.)

The heat denaturation and phenylalanine protection patterns of wild type and mutant enzymes are shown in Figs 2/*A*, 2/*B* and 2/*C*.

The heat denaturation of the enzyme follows first order kinetics without effectors with a rate constant of k_1

$$k_1 = \frac{1}{t} \ln \frac{E}{E_t} \qquad \text{Eq. (1)}$$

(where E and E_t are enzyme activities at times zero and t) whereas the denaturation rate constant of the enzyme-phenylalanine complex is k_2:

$$k_2 = \frac{1}{t} \ln \frac{EF}{EF_t}, \qquad \text{Eq. (2)}$$

(where EF and EF_t are the concentration of the native enzyme phenylalanine–complex at times zero and t).

If $k_1 = k_2$, it is possible to calculate the dissociation constant of enzyme–phenylalanine complex, K_i, from the apparent denaturation rate constant of the enzyme: k (Scrutton, Utter, 1965; O'Sullivan, Cohn, 1966)

$$\frac{k}{k_1} = \frac{K_i}{[\text{phe}]} \left(1 - \frac{k}{k_1}\right) + \frac{k_2}{k_1}, \qquad \text{Eq. (3)}$$

(where k is the apparent denaturation rate constant of the enzyme at [phe] concentration if [phe] $\gg$ [E]).

By plotting k/k_1 against $\frac{1 - \frac{K}{K_i}}{[\text{phe}]}$ the intercepts give k_2/k_1 and $\frac{\frac{k_2}{k_1}}{K_i}$ on the ordinate and abscissa, respectively, if $k_1 > k$, namely when phenylalanine stabilizes the enzyme.

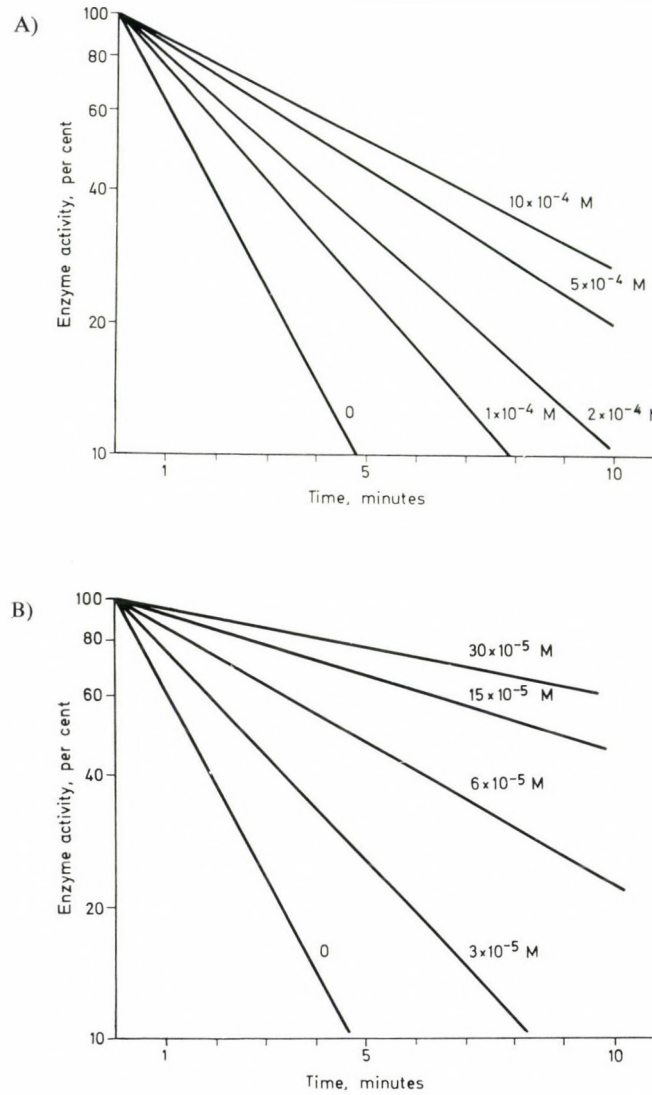


Fig. 2. Heat inactivation of DAHP synthases in the presence of phenylalanine. Samples of enzymes were incubated at 50° in 50 mM potassium phosphate buffer, pH 7.0, containing phenylalanine as indicated, *A*. Heat inactivation of wild type enzyme; *B*. Heat inactivation of DAHP synthase of mutant C25; *C*. Heat inactivation of DAHP synthase of mutant CH9. The broken line is calculated [phe] = 25×10^{-4} M

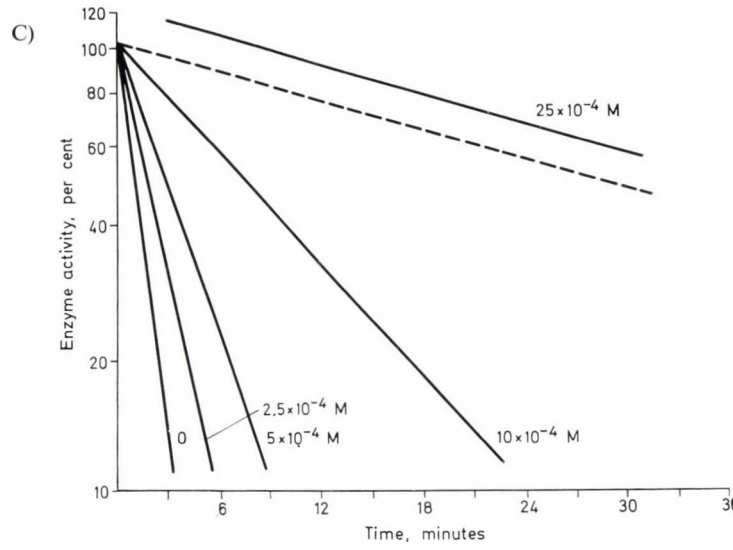


Table 1 summarizes the data on derepression, phenylalanine binding and inhibition.

Table 1

Repression and inhibition of mutant DAHP synthases by phenylalanine

Each mutant represents a group of mutants of similar properties with respect to repression, phenylalanine binding and inhibition

Strain	Number of similar mutants	Depression	Inhibition %	K_i
Wild	—	2.0 – 2.5	92	4×10^{-5}
C41	8	1.9 – 2.6	10 – 30	$1 - 4 \times 10^{-5}$
CC9	19	1.7 – 2.2	5 – 20*	$4 - 8 \times 10^{-4}$
C1	3	2.0 – 2.5	0	1×10^{-3}

* activation

Three groups of mutant enzymes were found. Enzymes of the first group bind phenylalanine and are sensitive to phenylalanine to a certain extent. Mutant enzymes of the second type are stabilized but not inhibited by phenylalanine, whereas members of the third group possibly do not interact with the amino acid.

Table 2 shows the activities and stabilities of the enzymes and enzyme–phenylalanine complexes at 50°. Two mutants had so labile enzymes that in crude extract we were unable to measure any DAHP synthase activity although these mutants overproduce phenylalanine in vivo. Similar results were found by Halsall and Catcheside (1971) with Neurospora.

In the case of wild type DAHP synthase (phe) and of some mutant enzymes, prepared by the acetone-hydroxyapatite method (Sasvári et al., 1971; Duda, Nagy, 1971), we applied Eq. (4) instead of (3):

$$\frac{k}{k_1} = \frac{K_i}{[\text{phe}]^n}\left(1 - \frac{k}{k_1}\right) + \frac{k_2}{k_1}, \qquad \text{Eq. (4)}$$

(where n is the Hill exponent of the enzyme).

Table 2

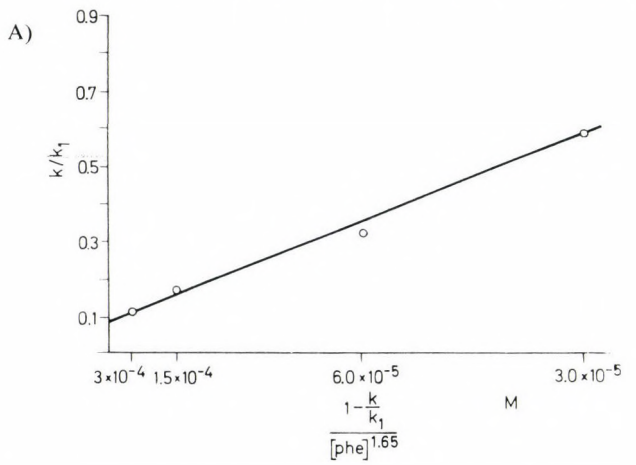
Enzyme content and enzyme stability of the mutants

Enzyme activities of mutants in per cent of wild type DAHP synthase (phe); and "half life times" of mutant enzymes and enzyme-phenylalanine complexes at 50°. Each mutant represents a group of mutants of similar properties with respect to enzyme activity, enzyme and enzyme–phenylalanine complex stability at 50°

Strain	Number of similar mutants	Enzyme activity %	Half life enzyme	Times, min EF complex
Wild	–	100	1.5	18.0
Cl/1	1	30–40	0.6	0.6
C10	12	30–40	4.5	15.0
CC23	7	50–60	0.6	6.0
Cl/7	2	20–30	2.0	2.2
C15	1	0	0	0

For wild type enzyme n is 1.65 (Duda, Nagy, 1971) and depending on the method of preparation it varies between 1.2 (Staub, Dénes, personal comm.) and 2.0 (Simpson et al., 1971).

A group of mutant enzymes behaves similarly in this respect (n varies be-



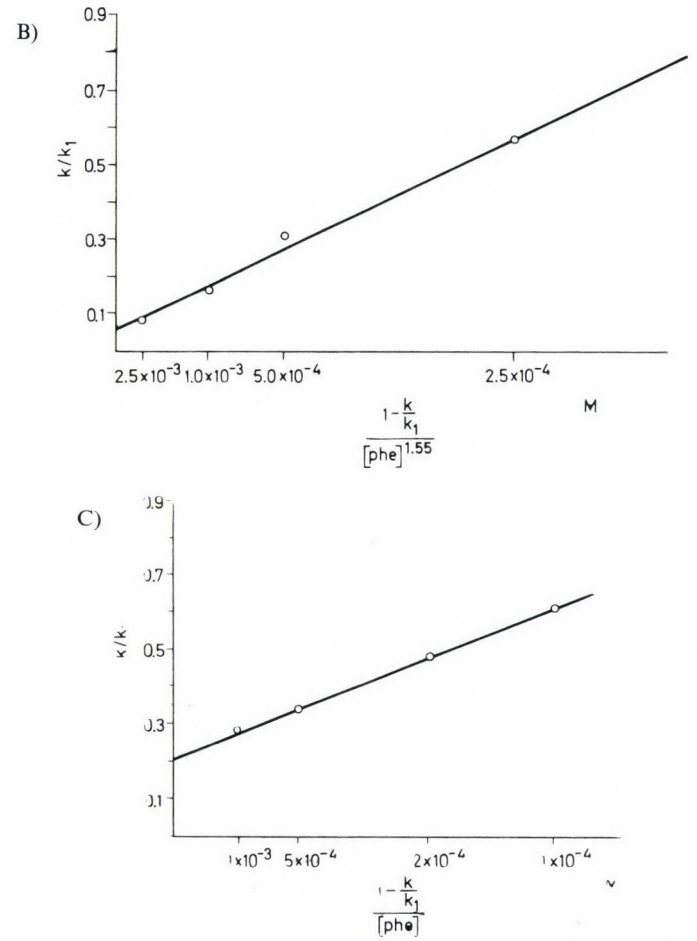


Fig. 3. Secondary plots of the first order rate constants of DAHP synthases. The slope of the curve equals K_i, the dissociation constant of enzyme–phenylalanine complex. *A*. Wild type enzyme. Hill's exponent of the enzyme 1.65; *B*. Enzyme of the mutant C19. Hill's exponent 1.55. *C*. Enzyme of the mutant CC5. Hill's exponent 1.0

tween 1.4 and 1.65), whereas the other group seems to show no subunit interactions (See Fig. 3/*A*, 3/*B* and 3/*C*).

In Fig. 2/*C* the denaturation curve of the mutant enzyme in the presence of 0.5 mM phenylalanine shows an interesting anomaly. Although the slope agrees with the calculated value the intercept with the ordinate is higher than 100%. This phenomenon also appears with some other mutants, and is probably due to a change in the structure of the enzymes produced at high concentrations of phenylalanine.

Mutant DAHP synthases (tyr) and (trp) that were activated and not inhibited by tyrosine and tryptophan were found by Halsall and Doy (1969) in Neurospora.

Summarizing, we can state that the enzymological study of feedback-insensitive DAHP synthase (phe) mutants has revealed different groups with respect to enzyme activity, stability in vitro at elevated temperature, phenylalanine binding, stabilization and inhibition by phenylalanine and subunit interactions.

We failed to demonstrate correlations between these groups (except that mutants which do not bind phenylalanine) thus mutants in group are similar only in respect of the properties indicated in the table. Furthermore, no direct evidence of simultaneous alteration of enzymological regulation and repression behaviour was found. (This, of course, may be due to the limited number of mutants.)

In the meantime the wild type DAHP synthase (phe) has been purified to homogeneity by Simpson et al. (1971) and by us (Duda, Nagy, 1971) by different procedures. The study of wild type and mutant enzymes might help to understand the molecular background of the allosteric behaviour.

We are indebted to Miss Ildikó Molnár, Mr Sándor Székely and R. Robert Hardi for their help.

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Estimation of Molecular Weights of Rat Liver Ribosomal Proteins

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Rat liver ribosomes were dissociated by EDTA and the subunits were isolated by sucrose density gradient centrifugation. The proteins of the subunits were analysed by electrophoresis in 10 and 12.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Altogether 26 to 28 electrophoretically different bands were separated from both subunits. The molecular weights of polypeptide chains present in the large and small subunits range between 6 000 and 70 000 daltons.

Introduction

In the last decade a great progress has been made in studying the proteins of prokaryote ribosomes, especially those of various strains of *E. coli* (Osawa, 1968; Kurland, 1970). All of the ribosomal proteins of *E. coli* have been separated and their molecular weights were determined by different methods (Traut et al., 1969; Hindennach et al., 1971a,b; Hardy et al., 1969).

Relatively little is known about the molecular weights of proteins from animal ribosomes. Hamilton and Ruth (1967) determined the average molecular weight of rat liver ribosomal proteins by equilibrium centrifugation. The molecular weights of individual protein components of rabbit reticulocyte ribosomes were also determined (Gould, 1970; King et al., 1971) by SDS-polyacrylamide gel electrophoresis. Recently Bielka et al. (1971) reported that the molecular weights of rat liver ribosomal proteins are mainly between 11 000 and 31 000, and that there are some components with higher molecular weights.

In the present paper we describe the analysis of the proteins of EDTA-derived ribosomal subunits from rat liver. The isolated subunits were treated with sodium dodecyl sulfate and subjected to electrophoresis in gels containing SDS, urea and 10 or 12.5% polyacrylamide. By this method molecular weights can be well estimated (Shapiro et al., 1967, 1970; Weber, Osborn, 1969; Dunker, Rueckert, 1969) with minimal risk to lose certain protein components during the preparation of the samples.

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Abbreviation used: SDS, sodium dodecyl sulfate.

Methods and materials

The preparation of ribosomes, ribosomal subunits and the preparation of proteins from isolated subunits for SDS-polyacrylamide gel electrophoresis were described earlier (Vereczkey et al., 1971; Hüvös et al., 1972). It was also shown that the isolated subunits did not contain any cross-contamination detectable by resedimentation in sucrose density gradient (Hüvös et al., 1972).

Samples containing 50 to 200 μg of protein were diluted with a buffered solution consisting of 0.05 M tris-HCl pH 7.0, 6 M urea and 0.1% SDS to a volume of 0.15 ml and after the addition of 2-mercaptoethanol in a final concentration of 2% the samples were immersed into boiling water bath for 4 minutes. After cooling, sucrose and bromophenol blue were added (approximate final concentration 10 and 0.001%, respectively) and the samples were applied to the gels.

The 1 cm long spacer gel contained 4% acrylamide, 1% N,N'-methylene-bis-acrylamide and 0.125 M tris-HCl, pH 7.0. The ratio of acrylamide to N,N'-methylene-bis-acrylamide was 29 to 1, in the 7 cm long separation gel. The diameter of the glass tubes containing the gels was 0.5 cm. The gels and electrode buffers contained 6 M urea in addition to the components described by Laemmli (1970).

In the first hour of electrophoresis a current of 1 mA per gel was used, then the electrode buffers were exchanged and the current was raised to 2.5 mA per tube. Electrophoresis was performed in a refrigerator at 5 °C for 5–6 hours. The gels were stained with 0.1% amidoblack 10 B dissolved in a mixture of 5 volumes of methanol, 5 volumes of water and 1 volume of acetic acid, and destained by washing with the same solvent mixture. Finally the gels were soaked and stored in 7% acetic acid solution. The relative mobilities were calculated according to Weber and Osborn (1969). Calibration curves (Fig. 1) for the determination of molecular weights were prepared by electrophoresing horse heart cytochrom C, bovine serum albumin, porcine insulin and pancreatic ribonuclease polymerized with diethyl pyrocarbonate as described by Wolf et al. (1970).

Diethyl pyrocarbonate was a product of Bayer Werke, Leverkusen. All other materials were obtained from Reanal, Budapest. Acrylamide and methylene-bis-acrylamide were recrystallized according to Loening (1967). Urea used for the preparation of samples and gels was deionized by mixed bed Elga resin (Elga Co., England) and then crystallized.

Results and discussion

The estimated molecular weights and their maximal deviations, calculated on the basis of analyses of proteins from five independent preparations and at least of two electrophoretic runs of each preparation, are summarized in Tables 1 and 2.

The sum of the estimated molecular weights listed in Table 1 is about 0.75×10^6 daltons. Since the RNA to protein ration in the 47 *S* subunit of rat liver ribosomes is 1.00 (Terao, Ogata, 1971) and the molecular weight of 28 *S* RNA is estimated to fall between 1.7 and 1.99×10^6 (Peterman, Pavlovec, 1963; Click, Tint, 1967), we may suppose that a number of these bands on the polyacrylamide gel consists 2 to 3 polypeptide chains having molecular weights indistinguishable

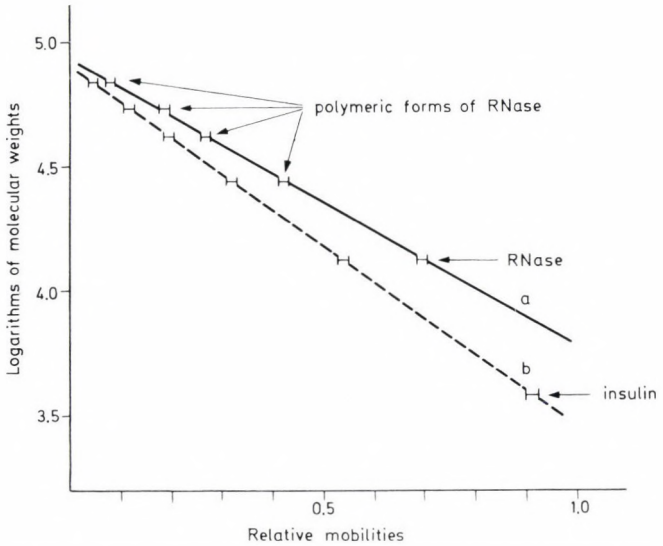

Fig. 1. Calibration curves for the determination of molecular weights by SDS-gel electrophoresis in gels containing a) 10, and b) 12.5% acrylamide. For further details see text. Ribonuclease polymerized by diethylpyrocarbonate and insulin were dissolved in SDS and urea and were subjected to electrophoresis as described in Methods. The horizontal lines indicate the relative mobilities of individual components measured on separate gels in repeated experiments

in these systems or that the large subunit of rat liver ribosomes contains more than one copy of certain proteins.

We can make a similar calculation in the case of small subunit, too. Summing up the molecular weights listed in Table 2, we get a value of 0.77×10^5 daltons. This value is in good agreement with that calculated on the basis of the protein to RNA ratio and the molecular weight of 18 *S* RNA. The 32 *S* subunit of rat liver ribosomes contains 55% protein (Terao, Ogata, 1971) and the molecular weight of its RNA is 0.7×10^6 (Peterman, Pavlovec, 1963; Click, Tint, 1967). That is, the protein moiety must be represented by 0.86×10^6 daltons. These results indicate that the small subunit of rat liver ribosomes contains 28 polypeptide chains. It should, however, be mentioned that we observed very great differences in the intensity of the stained bands. Some bands were visible in the region of high molec-

Table 1

Molecular weights of proteins from the large subunit of rat liver ribosomes

No.	Molecular weight estimated by electrophoresis on SDS polyacrylamide gel			
	10 per cent		12.5 per cent	
	Daltons $\times 10^{-3}$	± maximal deviation per cent	Daltons $\times 10^{-3}$	± maximal deviation per cent
1	69.1	4.9	70.1	4.0
2	64.0	1.4	64.2	1.2
3	61.1	1.3	62.0	3.2
4	53.1	1.1	54.5	2.8
5	50.9	1.8	50.2	2.4
6	46.2	1.3	46.3	2.4
7	42.5	1.9	43.8	1.8
8	39.5	3.8	38.6	3.1
9	37.1	2.7	37.4	1.3
10	32.4	2.5	32.7	2.1
11	29.1	3.1	29.6	4.0
12	25.4	1.5	26.3	3.8
13	24.4	1.6	24.5	1.6
14	22.5	4.0	22.6	1.3
15	20.0	4.5	20.8	3.8
16	18.6	4.8	18.2	1.0
17	17.2	1.2	17.4	1.1
18	16.5	1.8	16.6	1.2
19	15.6	1.9	15.3	3.3
20	14.4	3.5	14.3	1.4
21	13.2	4.5	13.3	1.5
22	11.8	4.2	12.1	3.3
23	10.5	1.9	10.2	2.0
24	9.5	5.0	9.3	3.2
25	8.6	3.5	8.4	4.8
26	7.0	1.0	6.7	1.5
27	—	—	6.0	3.3

ular weights only when large amounts of proteins were applied to the gels. Although staining with amidoblack 10 B does not allow one to estimate the amount of proteins present in the bands (Fazekas de St. Groth et al., 1963), the observation may suggest that certain polypeptide chains may be present in a ratio less than one per ribosome, and some other polypeptide chains which are identical or indistinguishable on a molecular weight basis may be represented by more than one copy per ribosome.

Our preliminary results indicate that the existence of these minor components is not due to the differences in the protein patterns of the subunits isolated

Table 2

Molecular weights of proteins from the small subunit of rat liver ribosomes

No	Molecular weight estimated by electrophoresis on SDS-polyacrylamide gel			
	10 per cent		12.5 per cent	
	Daltons $\times 10^{-3}$	± maximal deviation per cent	Daltons $\times 10^{-3}$	± maximal deviation per cent
1	70.6	2.4	69.2	4.8
2	65.6	1.8	65.0	3.1
3	62.4	1.1	61.1	4.8
4	56.2	1.8	56.2	2.3
5	53.3	3.8	52.5	2.9
6	48.5	1.2	47.7	2.1
7	46.0	3.0	44.2	3.2
8	40.7	2.8	39.9	1.7
9	33.2	4.5	33.8	3.8
10	30.6	1.9	31.0	4.5
11	27.3	3.3	27.6	4.7
12	24.4	2.9	24.2	3.2
13	21.6	3.7	22.6	3.1
14	20.4	2.4	21.7	0.9
15	19.8	5.5	20.0	2.5
16	17.4	1.5	17.4	2.3
17	16.4	3.0	16.4	1.2
18	15.5	1.9	15.3	1.3
19	14.5	2.8	14.6	1.4
20	13.5	2.2	13.5	2.2
21	12.6	2.4	12.3	1.0
22	11.7	2.6	11.8	2.7
23	10.7	2.8	10.8	4.6
24	10.0	1.0	9.9	1.0
25	9.2	3.3	9.4	2.1
26	8.4	3.6	8.8	3.4
27	—	—	7.9	2.5
28	—	—	6.6	0.9

from the free and membrane-bound ribosomes, since both subunits of these two kinds of ribosomes gave very similar, if not identical, protein patterns upon electrophoresis in the presence of SDS.

Recently Welfle et al. (1971) published the results of the fractionation of rat liver ribosomal proteins by two-dimensional polyacrylamide gel electrophoresis. They could separate 27 spots from the small subunit and 34 spots from the large subunit. These numbers are very near to those found in our experiments. Welfle et al. (1971) also claimed the existence of several minor components.

Comparing the estimated molecular weight of rat liver ribosomal proteins with those of the proteins from rabbit reticulocyte ribosomes (Gould, 1970; King et al., 1971), one can see that we found some bands revealing peptides corresponding to molecular weights larger than 57 000 and 39 000, which were the maximal estimated values for the large and small subunit of rabbit reticulocyte ribosomes, respectively. The range of molecular weights described in this paper is close to that published by Traut et al. (1969) for *E. coli* ribosomes. The distribution of the molecular weights, however, differs considerably. We found half of the protein bands from rat liver ribosomes in a region representing molecular weights larger than 20 000 daltons, whereas in the case of *E. coli* ribosomes only 30% of the proteins were found in this region.

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The Proteins of Rapidly Labelled Polysomal mRNP of Rat Liver

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Summary

Rat liver polysomes labelled with [^{14}C] — orotic acid for 40 min were incubated at 37° C and then treated with EDTA. The rapidly labelled and released, partially fragmented messenger-ribonucleoprotein fraction was separated from ribosomal subunits by sucros egradient centrifugation. Polyacrylamide gel electrophoresis of proteins of the messenger-ribonucleoprotein fraction showed that the electrophoretic mobility of one of the proteins was identical with that of component B of informofer proteins. Similarity could also be detected between the proteins of polysomal messenger ribonucleoprotein and informofer proteins on a molecular weight basis by means of dodecyl sulphate polyacrylamide gel electrophoresis.

Introduction

Little is known to date about the mechanism of transport of mRNA from the cell nucleus into the cytoplasm in eukaryotic cells. Newly synthesized RNA in the nucleus is bound to specific globular proteins, the so-called informofers (Samarina et al., 1968). The protein composition of nuclear ribonucleoproteins containing dRNA is relatively simple, three components can be detected by electrophoresis in polyacrylamide gel at pH 4.5, out of which the one encountered in the largest amount is denoted as component B (Samarina et al., 1968; Molnár, 1969). In the polysomes mRNA participating in translation is also in the ribonucleoprotein form, and this mRNP can be separated from the polysomes by EDTA treatment (Perry, Kelley, 1968; Henshaw, 1968; Cartouzou et al., 1969; Burny et al., 1969).

The treatment of polysomes with urea (Schweiger, Hannig, 1970), sodium deoxycholate or KCl (Olsnes, 1970) or with EDTA and KCl (Tomcsányi, Tigyi, 1971) results in the separation of a protein from the polysomes, the electrophoretic

Abbreviations: mRNP: messenger ribonucleoprotein; dRNA: RNA similar to DNA in base composition; EDTA: ethylendiamine tetraacetic acid, disodium salt; rRNA: ribosomal RNA; TCA: trichloroacetic acid; SDS: sodium dodecylsulphate; PCA: perchloric acid.

mobility of which is very similar to, or even identical with, that of component B of informofer proteins. It has been proposed that this protein is identical with the protein component of the polysomal mRNP.

The question can be raised, however, whether this protein obtained from the polysomes is indeed a component of mRNP. Since in rat liver polysomal mRNA represents a great number of RNA molecules of different size, the isolation of the pure form of polydisperse mRNP liberated by the treatment of polysomes with EDTA seems at present to be a difficult task.

If polysomes are incubated at 37° C before EDTA treatment, the mRNP is fragmented and in sucrose gradient these mRNP fragments sediment slower than the small ribosomal subunit (Olsnes, 1971). This observation renders it possible to separate the fragmented mRNP fraction from ribosomal subunits and thus to analyze the proteins directly.

In the present paper we describe the analysis of proteins of rapidly labelled and partially degraded polysomal mRNP from rat liver.

The study of proteins by means of polyacrylamide gel electrophoresis confirmed the assumption that the informofer-like protein that can be separated from the polysomes is a component of polysomal mRNP. The mRNP proteins can be resolved by SDS electrophoresis into several bands of different molecular weight, at least three of which agree in molecular weight with the individual components of informofer proteins.

Materials and methods

Materials

[6-^{14}C] – orotic acid (44.5 mCi/mmole) was purchased from the Radiochemical Centre (Amersham). All other chemicals were obtained from Reanal (Budapest). Synpor 2 celluloseacetate filters were procured from Chemapol (Prague).

Methods

Male CFY rats weighing 180–200 g were starved for 36 hours, then 20 μCi per 100 g body weight [6-^{14}C] orotic acid was administered to them intraperitoneally 40 minutes before killing. Livers were excised, rinsed with STKM (0.25 M sucrose, 0.05 M Tris-HCl, 0.025 M KCl, 0.005 M $MgCl_2$, pH 7.2) and the total polysome fraction was isolated by the slightly modified method of Blobel and Potter (1967), as described previously (Tomcsányi, Tigyi, 1971). The polysome pellet was suspended in TKM (0.05 M Tris-HCl, 0.025 M KCl, 0.005 M $MgCl_2$, pH 7.5), centrifuged at 3 000 r.p.m. for 10 minutes to remove aggregated material and the supernatant fraction was used as polysome preparation.

Polysome concentration was then adjusted to about 10 mg/ml on the basis

of the extinction coefficient for polysomes $A_{260}^{0.1\%} = 11.2$ (Breillatt, Dickman, 1966).

The polysome suspension was incubated at 37° for 30 minutes and then rapidly cooled down to 0°. EDTA (0.25 M, pH adjusted to 7.5 with KOH) was added to suspension to reach a final concentration 0.02 M higher than that of Mg^{++}, and the suspension was layered on top of a 28 ml 15–30% (w/w) linear sucrose gradient containing 0.15 M KCl, 0.02 M EDTA and 0.01 M triethanolamine, pH 7.5. The gradients were centrifuged in the 3 × 35 ml swing-out rotor of a Janetzki VAC 601 ultracentrifuge for 24 hours at 29 000 r.p.m. After centrifugation the tubes were punctured and fractions of 12 drops were collected. Two drops of each fraction were diluted with 0.15 M NaCl for the determination of absorbancy and afterwards 100 μg purified rat liver rRNA was added as carrier, the mixture was made up to 5% in TCA and the precipitate was collected on Synpor 2 filters and washed with 3% PCA. Radioactivity was determined in a Packard liquid scintillation spectrometer (Model 3380) in toluene-containing scintillation liquid which comprised 100 mg 1,4-bis(2-)5-phenyl(-oxazolyl)-benzene (POPOP) and 4 g 2,5-diphenyloxazol (PPO) per 1000 ml.

The zones of the gradient 21–17*S*, 15–11*S* and 9–5*S* were collected, the material of each zone was divided into two portions and was precipitated by the addition of TCA (10% final concentration). One part of each zone was used for polyacrylamide gel electrophoresis, the other for SDS polyacrylamide gel electrophoresis.

For polyacrylamide gel electrophoresis the precipitated material was dissolved in 0.5 ml buffer containing 6 M urea, 0.06 M potassium acetate and 10% sucrose, pH 6,8, and was dialyzed against the same buffer. Onto each gel a 0.2 ml-sample was applied. The disc electrophoresis was carried out at pH 4.5 according to Leboy et al. (1964) in the modification of Molnár (1969) for informofer proteins. The gels contained 7.5% acrylamide and 0.23% N,N'-methylene-bisacrylamide. With the exception of split gel electrophoresis, only the small pore gel was used (Hjerten et al., 1965).

For SDS polyacrylamide gel electrophoresis the precipitated material was dissolved in 0.5 ml buffer containing 0.01 M sodium phosphate, 1% SDS and 1% 2-mercaptoethanol, pH 7.0, and incubated at 37° C for 2 hours. The mixture was then dialyzed against a buffer containing 0.01 M sodium phosphate, 0.1% SDS and 0.1% 2-mercaptoethanol, pH 7. Onto the gels 0.2 ml samples were applied to which 1 drop of glycerol, 10 μl 2-mercaptoethanol and 10 μl 0.05% bromophenol blue were added. Electrophoresis was performed according to Weber and Osborn (1969) in 10% gels. The gels were stained with 1% amidoblack B 10 solution in methanol-acetic acid-water (5 : 1 : 5) mixture. Excess dye was removed with the same solution. For the determination of molecular weight pancreatic ribonuclease treated with diethylpyrocarbonate was used as standard (Wolf et al., 1970).

Informofer proteins were prepared from the 30*S* zone of the sucrose gradient according to Samarina et al. (1968).

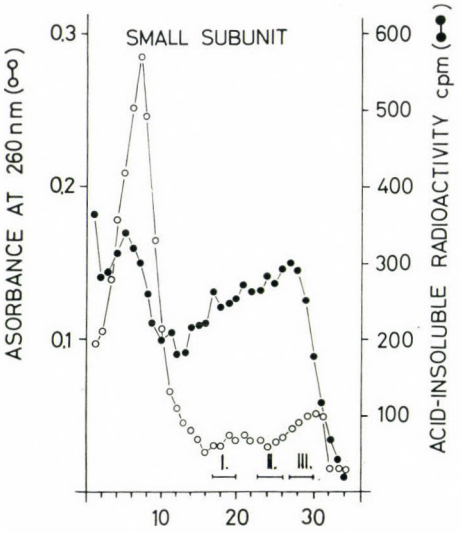

Fig. 1. Sucrose gradient centrifugation of rapidly labelled and fragmented mRNP. Rat liver polysomes (17 mg) labelled with [^{14}C]-orotic acid and prepared as described in Methods, were incubated at 37° C for 30 min and then rapidly cooled to 0°. EDTA was added to 0.02 M final concentration. Immediately afterwards the mixture was centrifuged in a 15—30% (w/w) sucrose gradient containing 0.15 M KCl, 0.02 M EDTA and 0.01 M triethanolamine, pH 7.5, for 24 hours in the 3×35 ml swing-out rotor of a Janetzki VAC 601 ultracentrifuge at 29 000 r.p.m.
Absorbance at 260 nm: ○——○
Radioactivity: ●——●
I: 21—17*S*; II: 15—11*S*; III: 9—5*S*.

Results

Sucrose gradient centrifugation of rapidly labelled polysomes incubated at 37° C and treated with EDTA

The incubation of polysomes at 37° C for 30 minutes produced 27—5*S* fragments of rapidly labelled mRNP. If the time of centrifugation is properly chosen so that the large ribosomal subunit should sediment, the mRNP fragments can be readily separated from the small subunit (Fig. 1). Although during the time period available for labelling 18*S* rRNA is also labelled (Greenberg, Penman, 1966), but with the small subunit radioactivity and light absorption do not run completely parallel, which suggests that a minor fraction of fragmented mRNP remained bound to the small ribosomal subunit (Olsnes, 1971).

Polyacrylamide gel electrophoresis of the proteins of rapidly labelled and partially fragmented mRNP fraction

The zone of the gradient lighter than the small ribosomal subunit was divided into three parts (Fig. 1) and collected. Since nuclear 30*S* dRNP particle and the

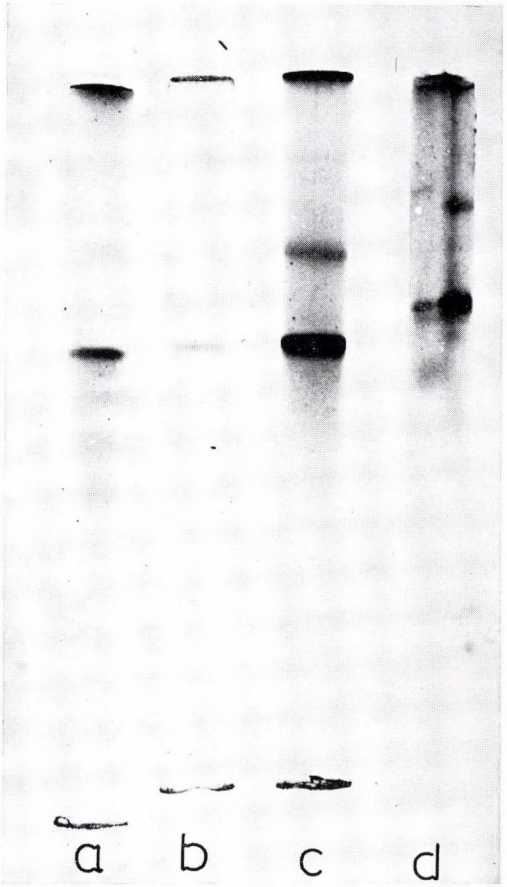

Fig. 2. Polyacrylamide discgel electrophoresis of the various fragments of rapidly labelled mRNP and of informofer proteins. The proteins were prepared and electrophoresed in 7.5% gels as described in Methods. Running time: 20 min, 3 mA/tube; 4 hours, 5 mA/tube. a) Proteins from zone I (21—17*S*) of Fig. 1. b) Proteins from zone III (9—5*S*) of Fig. 1. c) Informofer proteins. d) Split gel electrophoresis of informofer proteins and the proteins of zone I.

purified informofer protein give identical polyacrylamide gel electrophoretic patterns, (Molnár, personal communication), and since only small amounts of the material were available, the mRNP fraction obtained from the gradient was treated as described in Methods and was electrophoresed without removing RNA. Fig. 2 shows the gel electrophoretic patterns of mRNP from the various gradient zones and of informofer proteins. Among both the heavier (21—17*S*, Fig. 2 a) and the lighter (9—5*S*, Fig. 2 b) zone proteins there is one protein, the electrophoretic mobility of which is very similar to that of the informofer (Fig. 2 c). In the electrophoretic pattern of mRNP proteins derived from the 21—17*S*

zone there are, in addition to the informofer-like protein, four faster and one slower moving bands. It should be noted that part of the introduced material remained on the top of the gel.

In order to compare the informofer-like protein with the informofer, split gel electrophoresis was performed. As shown in Fig. 2 d, the electrophoretic mobilities of the informofer-like protein from the 21–17*S* zone and of the informofer are identical.

SDS polyacrylamide gel electrophoresis of proteins from the rapidly labelled and partially fragmented mRNP

Since the SDS polyacrylamide gel electrophoretic patterns of the 30*S* dRNP particle and purified informofer seem to be identical (Tomcsányi et al., unpublished observation), the mRNP material obtained from the various zones of the gradient was electrophoresed without removing RNA. The protein fraction derived from any of the three zones could be resolved into 17 bands by SDS gel electrophoresis (Fig. 3 a, b and c). The number and location of bands are identical in the case of the proteins of all three zones, but the intensity of the bands varies by zones. The most prominent band in zones I (21–17*S*) and II (15–11*S*) corresponds to a protein of molecular weight about 39 000 daltons. In zone III (9–5*S*), in addition to this 39 000 molecular weight protein, another of molecular weight 30 000, is also intensively stained.

The SDS electrophoretic pattern of informofer proteins reveals more than 10 bands (Fig. 3 d). The four most intensive bands correspond to 30 000, 34 000, 39 000 and 43 000 molecular weight proteins. The electrophoretic pattern is in accord, even as regards the molecular weights, with the SDS electrophoretic picture described by Niessing and Sekeris (1971a, b) for nuclear dRNP proteins.

Discussion

At present there are two opposing view in the literature concerning the nucleocytoplasmatic transport of mRNA. One of them, founded on the comparison of proteins obtained from the polysomal mRNP of rabbit reticulocytes (Lukanidin et al., 1971) and of immature duck erythrocytes (Morel et al., 1971) with the dRNP proteins of the nucleus, claims that the protein compositions of the two RNP's are different. Thus the protein component of nuclear dRNP, the informofer, participates only in the transport of mRNA from the chromatin to the nuclear membrane. The other notion, based upon experiments with rat liver, holds that the informofer proteins take part in the transfer of mRNA from the nucleus to the ribosomes. Ishikawa et al. (1972) observed that labelled nuclear RNA was bound to ribosomes together with labelled nuclear proteins.

Our results seem to support the latter idea. The electrophoretic mobility in polyacrylamide gel at pH 4.5 of one of the proteins of the mRNP fraction, which

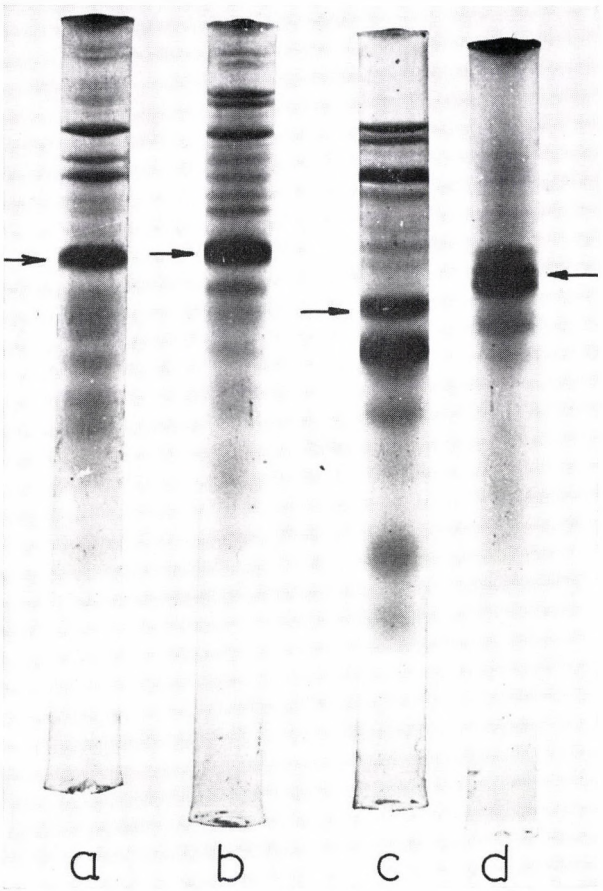

Fig. 3. SDS polyacrylamide gel electrophoresis of the various fragments of rapidly labelled mRNP and informofer proteins. The proteins were prepared and electrophoresed as described in Methods. a) Proteins from zone I (21—17*S*) of Fig. 1. b) Proteins from zone II (15—11*S*) of Fig. 1. c) Proteins from zone III (9—5*S*) of Fig. 1. d) Informofer proteins. a, b. an d: 8 mA/tube, 7 hours; c: 8 mA/tube 7.5 hours. Staining with amidoblack B 10 for 12 hours. 39 000 daltons molecular weight: →

was rapidly labelled and separated from ribosomal subunits by partial fragmentation, is identical with that of the B component of informofer proteins (Fig. 2). Thus the informofer-like protein released by various treatments of polysomes (Schweiger, Hannig, 1970; Olsnes, 1970; Tomcsányi, Tigyi, 1971) indeed represents one of the protein components of polysomal mRNP.

The protein fraction of rapidly labelled and partially fragmented mRNP can be resolved by SDS polyacrylamide gel electrophoresis into several proteins ranging in molecular weight between 15 000 and above 100 000 daltons. A protein

of molecular weight 39 000 daltons was encountered in the largest amount. This molecular weight agrees with that of one of the major component of informofer proteins. Some other bands, for example proteins of molecular weight 30 000 and 64 000, which occur in informofer proteins, can also be found among the proteins of polysomal mRNP.

As the patterns obtained by gel electrophoresis at pH 4.5 and by gel electrophoresis in SDS are different, it cannot be decided on the basis of our results how many protein components are encountered in polysomal mRNP. It seems probable, however, that the polysomal informofer-like protein could be resolved into several components by SDS electrophoresis, just like the informofer.

Since among the proteins of polysomal mRNP several can be found that are identical with informofer proteins, as far as molecular weight and electrophoretic mobility are concerned, we may conclude from our results that, at least in rat liver, some of the informofer proteins, e.g., the components of molecular weight 30 000, 39 000 and 64 000, can participate in the nucleo-cytoplasmatic transport and ribosomal binding of mRNA.

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Volume Doses Determined by Computer from Dose-distribution Measurements

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The work covered the experimental examination of the variation of volume doses with electromagnetic radiations of various energy in case of identical skin doses and nearly parallel beams corresponding to the cosmic space. Experiments were performed with a large-sized dog phantom made of a compound termed Mix-D and cut into slices by planes perpendicular to the direction of radiation. In the planes of section exposures were taken on films in order to determine dose distribution and small-size condenser ionization chambers were irradiated for direct calibration of the films. The densitometry of the films at the corner points of a square-centimetre network gave several thousands of measurements results for each sort of radiation, from which the volume doses falling upon the individual sections were determined with a computer by the reiterated application of the Mayneord formula (Jaeger, 1959). The measurements were performed at 250 cm focus-to-skin distance with ^{60}Co gamma radiation and with X-rays of three different quality: 200 kV and 0.5 mm Cu filter, 100 kV and 0.35 mm Cu filter, 70 kV and 1 mm Al filter.

Introduction

Research work in the field of cosmic biology and medicine started in Hungary in 1968 under the auspices of Intercosmos Cooperation established between the socialist countries (Várterész, 1971). It goes without saying that, in the first line, we could undertake only such investigations that could be performed under terrestrial circumstances, whose preliminary conditions were given for the best part and whose results could be put to use also under terrestrial conditions, primarily for planning radiation protection.

A job of this nature is the examination of the problem how volume doses will develop in animals and in the human body with electromagnetic radiations of various quality, in case of identical skin doses and parallel or nearly-parallel beams corresponding to cosmic space.

For the purposes of comparative examinations phantoms were made of a compound termed Mix-D (60 per cent paraffin, 30 per cent polythene, 6 per cent magnesium oxid and 4 per cent titanium dioxid) displaying the same absorptions and scattering properties as the soft tissues of human body. Namely, for the determination of volume doses with large phantoms a procedure linking up the calculation of volume doses to direct measurement results at as many points as

possible, appeared to be the most reliable since it excludes the potential error source which would occur in calculations starting from a single measurement result because of multiple scatterings and other effects difficult to follow by calculations, though unavoidable in thick media. The essence of our procedure consisted of determining dose distribution by direct measurements in planes perpendicular to the direction of radiation at several hundred points and of calculating on this basis the volume doses for the slices between two planes.

In this paper the measurements performed on the first large-size dog phantom lying prone and the calculations that followed them will be discussed.

Experimental

For dose measurements a combination of film dosimetry and of the Sievert condenser ionization chamber technique seemed the most suitable method. It consisted in films packed in black paper being exposed to radiation in the planes of section, in order to obtain precise dose distributions, plus small-size condenser ionization chambers being placed at the planes of section for the determination of exposures with high precision, i.e., for the calibration of the film dosimeters in every case. The latter is of particular importance for irradiations along the long axis when, owing to multiple scatterings, the composition of the radiation spectrum undergoes significant variations. Therefore, the light-sensitive emulsion of great energy dependence needs direct calibration at all depths.

Comparative tests were performed with ^{60}Co gamma radiation and with three X-ray radiations of different energies: 200 kV and 0.5 mm Cu filter, 100 kV and 0.35 mm Cu filter, 70 kV and 1 mm Al filter. The maximum source-to-skin distance was determined by the dimensions of the treatment rooms, it was invariably 250 cm.

For irradiation perpendicular to the long axis the dog was sliced by three horizontal planes. Two 24 by 30 cm Ferrania Sigma-type industrial X-ray films were fixed to each surface of section. Every section surface was irradiated for optimum exposure times previously determined experimentally in order to obtain film densities that could properly be measured with our densitometer.

In addition, a standard calibration series with various exposures, were prepared for every film pair; for the X-rays under a phantom plate 5 cm in thickness at a distance of 105 cm and with various exposure times, and for ^{60}Co a stepped lead shield was applied. A 3 cm thick Mix-D phantom plate was inserted between the stepped lead shield and the film. A thick Mix-D plate was placed under the calibrating films as a backscattering medium in every case. The light source of the densitometer operated with a battery. Densitometry was performed at the corner points of a network of 1 cm^2, by the transillumination of surfaces of about 1/6 cm^2.

The small-size condenser ionization chambers (Bozóky, Rodé, 1955) used for the measurements of exposures were moulded of air-equivalent bakelite, without handles (Fig. 2). The inner graphite electrode was carried by amber in-

Fig. 1. Dog phantom prepared of compound Mix-D for irradiations perpendicular to the long axis

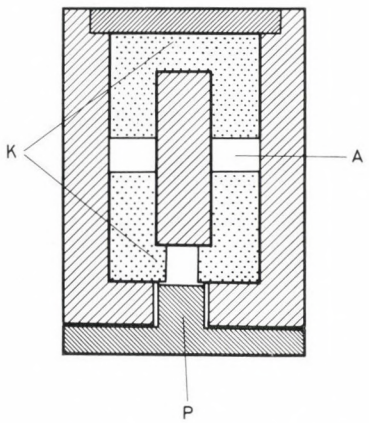

Fig. 2. Small-size condenser ionization chamber of 6 mm^3 air volume

sulators. The inner air space of the cylindrical ionization chambers, 7 mm in external diameter and 10 mm in external length, was not more than 5–6 mm^3 and thus the chambers permitted nearly point-like measurements. The small chambers were calibrated in the X-ray range with the standard R-meter free-air chamber of our Institute (Bozóky, 1940), and in the gamma- radiation range on the basis of the standard measurements performed in the Institute by the Vienna

Laboratory for Dosimetry of the International Atomic Energy Agency (Bozóky, et al. 1973).

The method outlined above yielded some 4000 measurements for volume-dose determination for each type of irradiation.

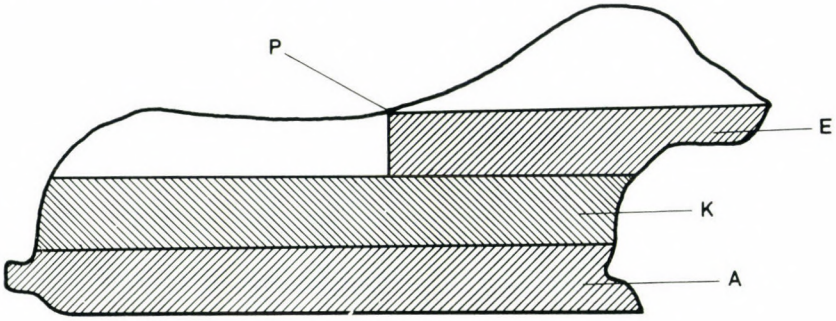

Fig. 3. Cross-section of the dog phantom

Calculation of volume doses

The volume dose of the dog phantom is composed of two parts:

$$D = D_1 + D_2 , \tag{1}$$

where D_1 = the volume dose of the parts hatched in Figure 3, and D_2 = that of the rest of the phantom.

Calculation of D_1. D_1 was calculated from the data of densitometry by the use of the Mayneord formula. As seen in Figure 3, D_1 is the sum of the volume doses of the three sections:

$$D_1 = D_A + D_K + D_F , \tag{2}$$

where, e.g., D_A may be written as

$$D_A = D_{A'} - D_{A''} . \tag{3}$$

$D_{A'}$ is the volume dose from the top of the section to full absorption, $D_{A''}$ that from the bottom of the section to full absorption.

$D_{A'}$ can be calculated by the use of the Mayneord formula as follows

$$D_A = \sum_{i=1}^{22} \sum_{k=1}^{65} 1.44 \cdot F \cdot q \cdot D_{A,ik} \cdot d_{A,ik} \left[1 + \frac{2.88 \cdot d_{A,ik}}{f} \right] \cdot \left[1 - e^{-\frac{z}{d_{A,ik} \cdot 1.44}} \right] \tag{4}$$

where

$F = 1$ cm^2,

q = normalizing factor to normalize the formula for unit irradiation dose on the basis of the exposure measured at point P of Figure 3. By the use of this

factor one can ensure a condition as if all films were placed in the dog at the same time and unit dose were delivered at point P,

$D_{A,ik}$ = exposure measured at the point defined by the number pair i,k, worked out by the computer from the function obtained from the density calibration series for each point of densitometry. The functions for the various film and radiation types were given as a number of straight-line equations,

$d_{A,ik}$ = the thickness of the half-value layer belonging to the point i,k of the section A, picked out by the computer from the half-value thickness versus material thickness before the layer functions according to the indexing,

z = thickness of the section,

f = source-to-section distance, longer than, or equal to, 250 cm, and finally

the exponential term takes into account the element $D_{A''}$ of Formula 1.

D_K and D_F were calculated along the same lines.

Calculation of D_2. To determine D_2, a procedure corresponding to the definition of volume dose was adopted. The volumes of some thinner slices of the two phantom parts in question (those left blank in Figure 3) were measured, the results multiplied by the average doses calculated for the individual elements, and the products summed. Volume measurements were performed in a way that while the planes of section were held horizontal, the phantom part was immersed with great caution as deep as were the marks previously made on a pot filled with water to repletion, and the volumes of displaced water were measured.

The results of the volume dose determinations for the four types of radiation, normalized for 1 R exposure applied to point P, have been compiled in Table 1.

Table 1

Volume doses transformed for 1 R surface irradiation in case of irradiations perpendicular to the long axis of the dog

kV	Filter	Volume doses	Relative values
70	1 mm Al	5.600 gR	1.0
100	0.35 mm Cu	7.700 gR	1.4
200	0.5 mm Cu	9.800 gR	1.8
^{60}Co	—	10.800 gR	2.0

Conclusions

1. As seen in the Table, in the case of identical skin doses the volume dose applied to the dog increases with increasing quantum energy of the radiation: with radiation of 100 kV the volume dose is 1.4 times, with that of 200 kV 1.8 times, and with gamma radiation twice as high as with radiation of 70 kV.

2. When applying the same irradiation along the long axis of the dog the volume doses significantly drop partly because, in the plane perpendicular to the direction of the radiation, the body cross-section considerably decreases and, partly because, owing to the increase of body thickness, also the proportions of the volume doses change: as the energy of radiation increases, the volume-dose proportions with respect to the softest radiation also increase. With 100 kV radiation to a negligibly small extent only, with 200 kV radiation by 6 per cent, and with gamma radiation by 30 per cent.

3. This increase, however, can compensate only for a small fraction of the drop of the volume dose even with gamma radiation. Thus, it is the decrease of cross-section in the plane perpendicular to the direction of radiation which is critical for the drop of volume dose.

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A Simple Method for Dose Measurements in a Biological Irradiation Facility

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The influence of reactor poisoning and burning up on the dose rate was investigated in mice irradiated in a biological irradiation facility operated at the research reactor in the Central Research Institute for Physics, Budapest. A simple method based on continuous monitoring is described which permits a more precise planning of experiments and their intercomparison than either activation technique or ionization method.

Introduction

In the vicinity of $LD_{50/30}$ (Sántha et al., 1972) the survival vs. dose curve for neutrons is rather steep. Therefore, a standard error of about 10–15 per cent is somewhat high for certain biological experiments. However, in mixed neutron-gamma field no better results could be obtained so far either with activation technique (Zaránd et al., 1972c) or ionization method (Aglincev, 1961). This is a serious shortcoming, as the majority of our experiments are carried out in more than one reactor period, requiring a continuous intercomparison. The choice of a biological test other than survival is possible but without survival it is inadequate for the examination of the radio-protective effect of various compounds. Therefore, the aim of this paper is to find a better and easier dosimetrical method based on a monitoring system.

Methods

In a previous paper the author described a monitoring system and came to the conclusion that the dose-rate stability was better than ±4 per cent *within the same reactor periods*. The error due to reactor poisoning is also included in this figure. In the paper referred to it has been assumed that the reactor spectrum does not change in the given reactor period and monitor counting rate is a linear function of dose rate. This assumption does not hold necessarily for different core configurations, as the same effective neutron fluence may correspond to different doses due to spectrum changes. As a first step, the measured activities of different threshold detectors were converted to kerma spectrum and kerma rate by using a computer code (Zaránd, 1972b).

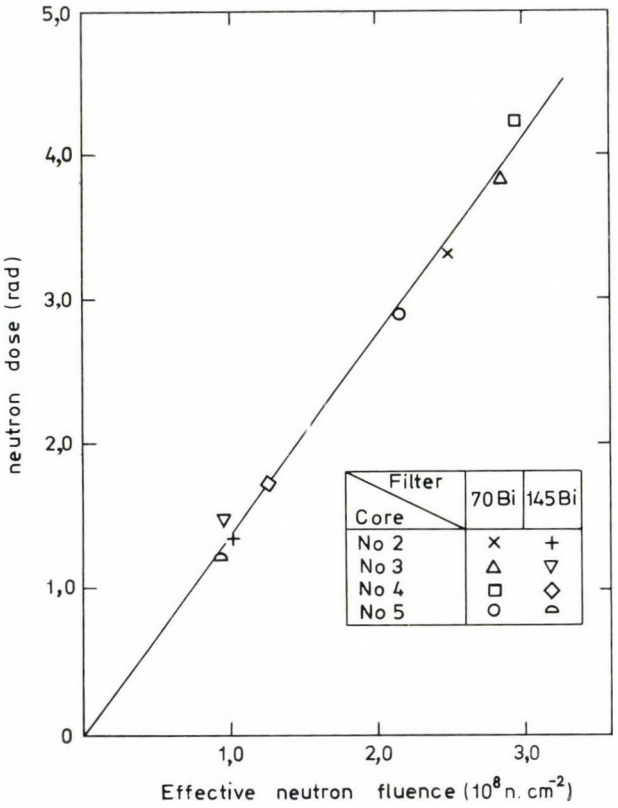

Fig. 1. Effective neutron fluence measured with S ($E_{\text{eff}} = 3$ MeV; σ_{eff} 0.3 barn) vs. neutron dose absorbed in mice

Results

The absorbed-dose rate in mice was computed from the kerma rate (Zaránd et al., 1971). The results of these calculations are shown in Fig. 1. All of the measured points fit in well with a straight line, though the dose rate values were measured by using different filters in various core configurations. This result indicates that the spectrum changes are not important and the neutron dose may be monitored. Along with our previous findings [gamma contaminations are characteristic of a filter and constant within limits of experimental errors (Zaránd, 1973)] this means that both gamma- and neutron-dose rates may be determined on the basis of monitor counting rate. It has to be mentioned that the reflector thickness in front of the biological channel remained unchanged during these investigations.

The second problem to be discussed is the contribution of dose rate changes resulting from reactor poisoning to the mentioned ± 4 per cent error. This problem

was investigated in four consecutive reactor cycles (about two months reactor operation) by our G-M counter monitoring system, using all of the filters. Different symbols in Fig. 2 refer to different reactor cycles. The measurements were normalized as follows:

$$I_{n,i} = I_i/[\sum I_i(t_{i+1} - t_i)/t_c]$$

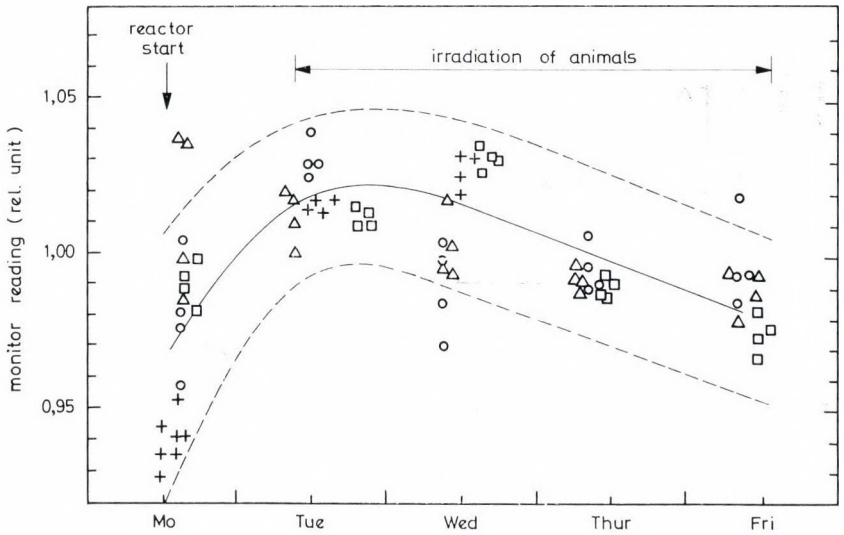

Fig. 2. The effect of reactor poisoning on neutron-dose rate measured by a G-M counter monitor

where I_i represents the counting rate measured at t_i, $I_{n,i}$ its normalized value and t_c the time of reactor operation in the cycle considered. The standard error of the points is $\pm(1.2-1.8)$ per cent depending on different counting rates for different filters. The Figure clearly demonstrates the influence of reactor poisoning on dose rate but the error due to this effect may be eliminated by continuous monitoring.

Fig. 3 shows the effect of reactor burning up on dose rate. All points are cycle averages referred to unit MW reactor power. Different core arrangements and average reactor power are also indicated in this Figure. The numbering of the core configurations is the same as in our contribution at the Teheran Symposium (Zaránd et al., 1973) but differs from that in the paper published in the Acta Biochim. et Biophys. (Zaránd, 1972a). Core configuration No. 4 corresponds to maximum loading and, consequently, maximum burning up, while a minimal

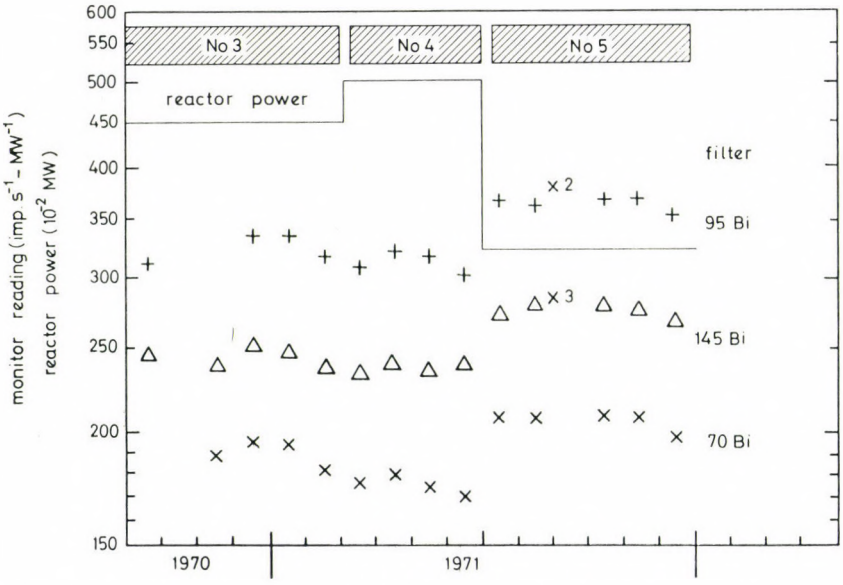

Fig. 3. The effect of burning up on neutron-dose rate. Core No. 4 corresponds to maximal burning up

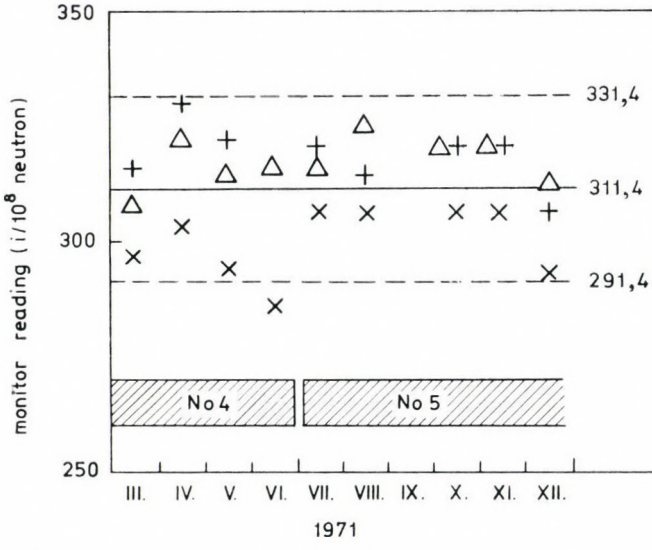

Fig. 4. Neutron dose relative to effective neutron fluence in core arrangements corresponding to maximal and minimal burning up

loading is used in core configuration No. 5. Between the two core configurations a step of about 20 per cent in the monitor reading vs. time curve is observable which, naturally, disappears if monitor reading per effective neutron fluence is plotted (Fig. 4), both quantities being dependent on each other.

Last but not least, both neutron- and gamma-dose rates (absorbed in mice) can be calculated by the following equations:

$$\dot{D}_n = I \cdot 3.13 \cdot 10^5 \cdot 1.37 \cdot 10^{-8}$$

$$\dot{D}_p = k \cdot \dot{D}_n$$

and the dose rates are obtained in rad/s if I is measured in count/s. The conversion factor k has a value of 0.584 and 0.262 for 70 and 145 mm Bi filters, respectively. $3.13 \cdot 10^5$ is the counts-to-fluence and $1.37 \cdot 10^{-8}$ the fluence-to-dose conversion factor.

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Electrons, Molecules, Biology and Cancer*

By

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Biology is dominated by the molecular concept, is a "Molecular Biology". I could never believe that the wonderful speed and subtlety of biological reaction could be brought about by clumsy closed shell macromolecules without the participation of much smaller and more mobile units, which could be no other than electrons. But electrons, to be mobile, demand a conductor. This made me suggest, more than thirty years ago, with my young friend and associate K. Laki, that proteins may be semiconductors. My proposition was unanimously rejected. It was pointed out to me that a simple look at proteins should convince me of my error because proteins were colorless and transparent, while semiconductors are likely to be colored and opaque.

To see whether calculations could lead somewhere, my young associate, J. Gergely, went to Leeds, England, to search the guidance of M. G. Evans. The calculations showed that energy levels, in protein, may be crowded into energy bands. The question, whether electrons, in such a system, are mobile, depends on their number. If there were twice as many electrons as underlying units, then al allowed places would be occupied creating a situation similar to that in an box, completely filled with marbles: there could be no mobility. This seemed to be the situation in proteins. To make the electrons or marbles mobile, some of them would have to be taken out.

The simplest way to take electrons out of a filled band is to excite them to the next higher empty energy level. If the two levels are so close together that even heat agitation can do this, then the substance is a semiconductor. The distance separating the two levels in protein was measured and calculated and was found to be rather great, several electron volts, and there is no energy in the living cell which could lift electrons through such a wide "forbidden zone". So I had to ask whether, perhaps, substances were present in tissues which could act as "electron acceptors", taking electrons out of the energy bands of proteins? This possibility was raised the following year by L. Brillouin to a comprehensive theory, according to which "acceptor impurities" make proteins into semiconductors. However, in the great number of proteins, isolated and thoroughly studied, no trace of such "acceptor impurities" was found and the theory remained rejected.

* Lecture delivered before the Hungarian Academy of Sciences on October 15, 1973.

I was so deeply convinced of the semiconductivity of proteins that for me the problem was: where was science misled? It was a puzzle. The solution is simple. Science missed protein semiconductivity because it forgot that proteins can, grossly, be divided into proteins which build structures and proteins which do not. This difference is important because the great biological functions, like motion, secretion or reflexes are all produced by structures. We know life from death by these functions which, essentially are all energy transformations, transductions of chemical energy into work, be this work mechanic, electric or osmotic. Since transductions are performed by structures, and it is these transductions which demand mobile electrons, we can expect semiconduction to be found in the structures. These structures are catered for by an extensive system of single soluble protein molecules which perform, so to say, the household chores around the structures, providing them with chemical energy. These soluble proteins, like serumalbumin, or various enzymes are easily accessible in quantity in molecular dispersion, while the molecules built into structures can rarely be extracted without denaturation. So what happened was that researchers wanting to study proteins, limited their work to the soluble proteins, like serum albumins or enzymes, which can easily be extracted from tissues, and called the extracted structures "residue" and sent them down the sink. The result is that protein chemistry, to its greatest extent, is a "chemistry of soluble proteins", and so semiconduction, which is a property of structures, has been overlooked.

When embarking with Jane McLaughlin on this line, our first luestion was: are structure-forming proteins also colorless? To answer this question, we blendored liver in a great excess of water, washed out the blood, precipitated the the proteins isoelectricylly and dissolved them in laurylsulfate. The resulting suspension is shown in Fig. 1. (colour plate). It had the color of Swiss chocolate, was dark brown, almost black, and was opaque, which suggested delocalized electrons.

The problem we were facing was a rather fundamental one: how far is life a molecular or an electronic phenomenon? Trying to answer such a basic question, one does well to satrt at the beginning and go back to tbe origins.

Life originated on a dark and airless globe, covered by dense water vapor. There was no light and no oxygen. We have no information about life in that period and can only theorize that under those poor conditions life could develop but the simplest forms and achieved continuity by proliferating as fast as conditions permitted, proliferation having been favored by a semiliquid state and simple make-up. Energy could be produced only by fermentation which demans no structure. This state I will call "State I" the main properties of which are summed up on the left side of Table 1.

As our globe cooled, and the water vapor condensed, light could get through. Life learned to catch its photons, with the energy of which it decomposed water into its elements hydrogen and oxygen. It linked the H to carbon, building foodstuffs, letting the O_2 go into the atmosphere. To recover the energy invested, the process had only to be reversed. With the oxygen, thus formed, the products

of fermentation could be oxidized and made to release their total energy, much of which was lost before. With this new and rich source of energy life could develop, differentiate and build complex structures with complex functions. The whole nature of the living systems changed. The semiliquid state was exchanged for a semisolid one. Complex mechanisms were built, performing complex functions. It was at this stage that semiconduction and free radicals had to be developed. This new state I will call "State II". Its main characteristics are summed up on the right side of Table 1.

Table 1

State I. (Anaerobic)	State II. (Aerobic)
Undifferentiated	Differentiated
Proliferation	Regulation
Semiliquid	Semisolid
Dielectric	Semiconductant
Fermentation	Oxidation

To induce these changes, the nature of protein had to be changed, and the question arises how could oxygen do this? O. Warburg made the wealth of energy responsible. Certainly, nothing new could have been built without energy, but energy, as such, cannot build anything. Energy can drive your car but not build it.

Oxygen owes its central role in biology to its ability to oxidize, that is, take over the electrons of other substances. The cells use it to oxidize their foodstuff with it. Could it then be that some of the oxidative metabolites contained oxygen in a form in which it still could take up electrons and thus take electrons from proteins, desaturate their energy bands, turning proteins into semiconductors? The products of oxidative metabolism contain various atomic groups which contain oxygen, and there is one among them, the C=O, the carbonyl, which can still accept electrons, one of the two bonds linking C to O being a π bond which has an empty (antibonding) orbital.

The C=O group is too small to accommodate easily a whole additional electron, but there is a way to extend its electronic system: by placing a second double bond next to it, either in the form of another C=O or in form of a C=C. The two double bonds being conjugated, their π systems fuse to a wider π system which is a good electron acceptor. This leads to two groups of good acceptors, the one containing O=C−C=O, a dicarbonyl, the other containing O=C−C=C, as an α-β unsaturated aldehyde. The simplest dicarbonyl is glyoxal, and the simplest derivative of glyoxal is methylglyoxal (Fig. 2), while the simplest α-β unsaturated aldehyde is acrylaldehyde, the simplest derivative of which is croton aldehyde (Fig. 2). These substances could easily be formed in biological oxidation. Can they, then, transform the protein into a semiconductor? I will simply call

them summarily "carbonyls", meaning thereby both the dicarbonyls (derivatives of glyoxal), and the α-β unsaturated aldehydes.

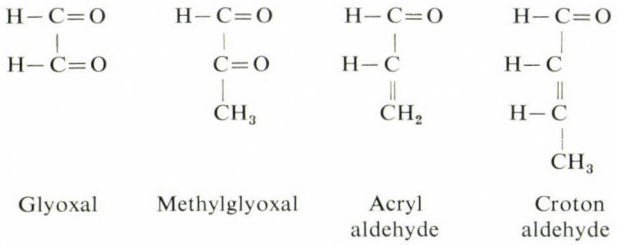

Fig. 2

What made the problem most exciting at this point was that for sixty years we knew that all living cells contain a most active enzymic system, the "glyoxalase" which could transform the reactive glyoxal derivatives into inactive hydroxyacids, transforming, for instance, methylglyoxal into lactic acid. The biological function of this enzymic system is unknown. Nature does not indulge in luxuries and so this enzyme had to have something important to do, but nobody knew what. Nobody could find glyoxal derivatives in tissues which could explain the function of this enzyme. Could, then, glyoxal derivatives not act as electron acceptors for proteins, desaturating energy bands and transforming thus proteins into semiconductors, bringing thus the cell into *State II*? The glyoxalase, then, could bring the cell back into *State I* by decomposing the regulatory glyoxal derivative, if, for some reason, such a transformation was needed.

The question which had to be asked first was: can glyoxal derivatives or α-β unsaturated aldehydes transform the proteins into semiconductors? If color can be taken as a sign of free radicals and semiconduction, then this question can easily be approached: we only have to incubate protein with one of these acceptors and see whether they develop color. What happens is shown in Fig. 3, (colour plate) in which the left beaker contains a 5% caseine solution (pH 7.4), while the right one contains the same caseine solution after incubation with 0.1 M croton aldehyde. The latter solution has assumed the red-brown color of liver. In the spectroscope it yielded a curve with a hump (Fig. 4/A). That electrons were transferred from protein to aldehyde, and the hump was due to the free radicals formed, was indicated by the electron-spin-resonance signal obtained by Dr. H. Kon. At pH 11 radicals are very unstable and there is no hump (Fig. 4/B). The differential of curve A and B gave curve C, a single structureless peak, which was responsible for the hump, and was characteristic for "charge transfer". Charge transfer can thus take place between proteins and carbonyls, in which a whole electron is transferred from protein to carbonyl in the ground state. Colered protein is produced in this transfer. The colored protein can be precipitated unchanged, the charge transfer complex being stable. This complex represents a new type of particle, which, in the case of caseine is built of a positive polyvalent radical of

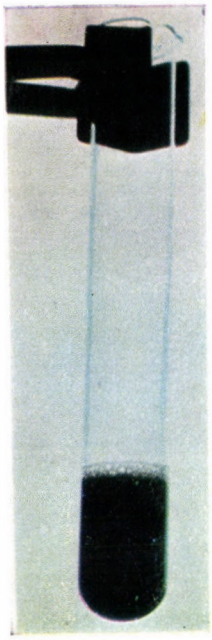

Fig. 1

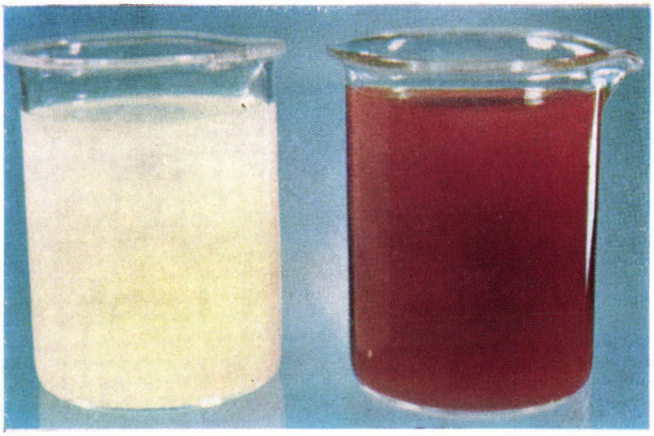

Fig. 3

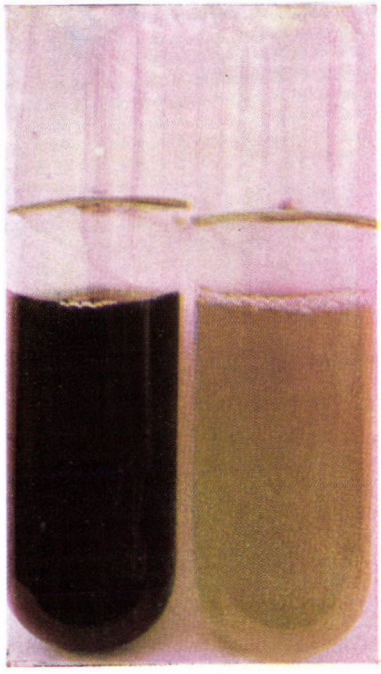

Fig. 9

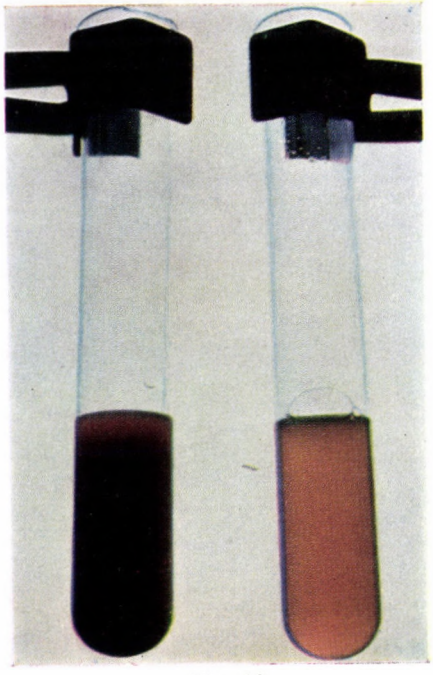

Fig. 10

Fig. 11

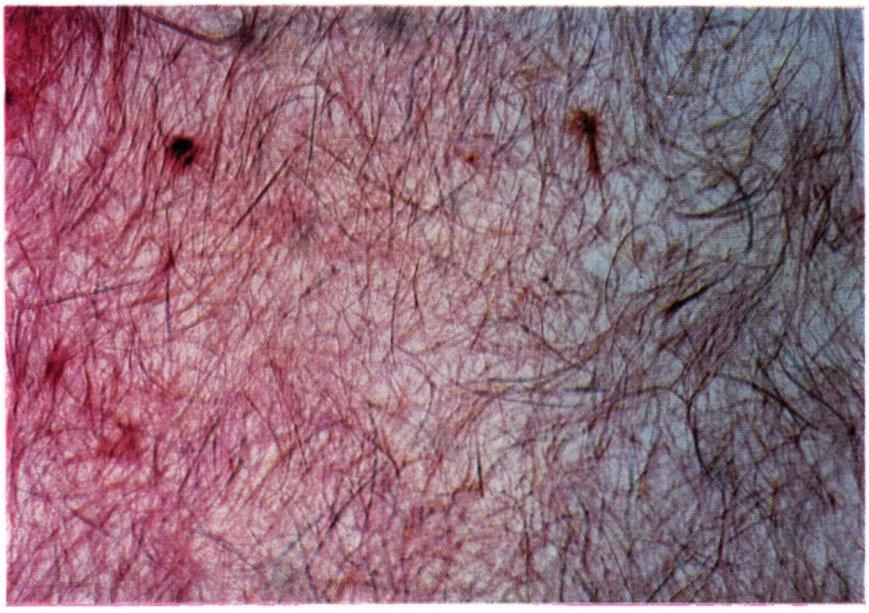

Fig. 12

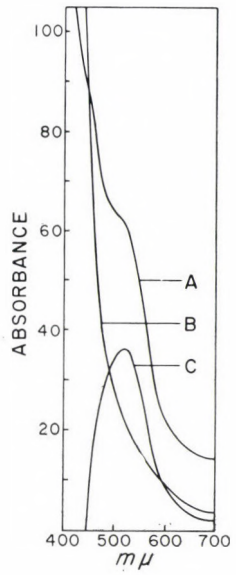
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Fig. 4

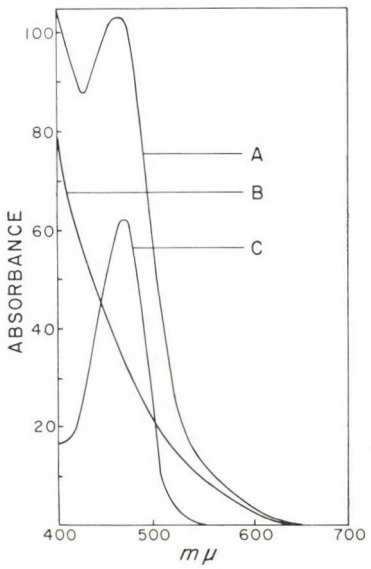
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Fig. 5

caseine and negatively charged radicals of the aldehyde. The complex had little tendency to dissociate, being held together strongly by the electrostatic attraction of the opposite charges. In spite of these intimate relations, the spins of the electrons were uncoupled. The transferred electron, and its hole were separated.

The electron which is transferred from protein to aldehyde could be no other than a non-bonded electron of the amino nitrogen. That this is so could be shown by the interaction of simple amines, like ethylamine, and carbonyls. These complexes are similar to the caseine complex. This can be illustrated by the interaction of ethylamine and methylglyoxal. Owing to instability of the radicals the complex has to be made, in this case, at $-25\,^{\circ}C$. At this temperature, in the spectroscope, again a curve with a hump was obtained (Fig. 5/A). The radical being unstable, at room temperature, no such hump was formed (Fig. 5/B). The differential of the two curves again yielded a sharp structureless peak (Fig. 6). A number of such complexes has been prepared with different amines and amino acids and carbonyls; they all showed similar spectral properties and electron-spin-resonance signals.

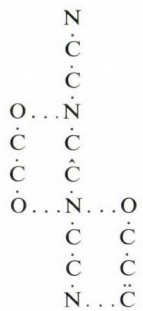

Fig. 6

The Fig. 6 shows how nicely, the protein and the discussed acceptors fit together. The middle row shows the sequence of atoms in the peptide chain, the row on the left the sequence in methylglyoxal. It can be seen how the carbonyl oxygens fit to the amino nitrogens of the protein, opening the possibility of overlap of orbitals and charge transfer on both ends. The row on the right side represents the atomic sequence in an unsaturated aldehyde. In this case the carbonyl oxygen fits one amino nitrogen, while the β carbon atom of the C=C double bond fits the other. This β carbon atom is strongly activated as witnessed by its ability to make additions.

These relations make it possible for the dicarbonyls or unsaturated aldehydes to attach themselves to the polypeptide chains of proteins. Since their two double links are conjugated, the electrons of their extended π systems are highly mobile. The dicarbonyls or the unsaturated aldehydes can form thus a conducting connection between the amino nitrogens offering an additional pathway for electronic mobility.

Such a protein molecule with attached ketone aldehydes or unsaturated aldehydes forms a very remarkable structure, its kernel, the peptide chain being a polyvalent positive free radical with desaturated conducting energy bands. Attached to this free radical are the negative free radicals of the carbonyls. Biochemistry, hitherto, was reluctant to assume the existence of stable free radicals within cells, free radicals being most reactive and having a net charge. The described structures, consisting of equivalent positive and negative radicals, have no net charge and no outward reactivity.

Another possible link between polypeptide chains and carbonyls is represented in Fig. 7. Here the carbonyls is thought to connect the amino groups of two

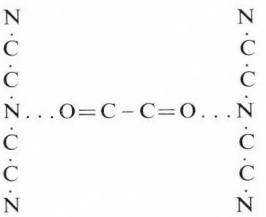

Fig. 7

peptide chains, cross-linking them, which must make the protein insoluble. It is easy to show that carbonyls actually do make proteins insoluble. Gelatine, for instance, incubated with 0.1 M glyoxal becomes insoluble; its solution can be melted no more. It seems likely that the dicarbonyls, and the unsaturated aldehydes are used by nature for different purposes, the first producing changes which can rapidly be cancelled by the glyoxalase which inactivates the dicarbonyls, while unsaturated aldehydes may produce more permanent changes, additions being poorly reversible. These latter may be inactivated by methylation which converts the aldehyde into a less reactive ketone.

It seems likely that structures are built by cross-linking peptide chains by carbonyls, which also explains why only structure proteins are colored, both the structure formation and color being due to the carbonyls.

The carbonyls can desaturate the energy bands and form conducting connection between peptides and amino groups only if reacting with the amino groups of the peptide chain, and not with the amino groups of the side chains. Arginine was found to be rather unreactive towards dicarbonyls, while caseine, which had the amino groups of its lysine side chain methylated, showed the same reactivity as un-methylated caseine. Polyglycine, which had no side chains at all, showed a reaction similar to proteins.

Two general remarks may be made here. Charge transfer, hitherto, has been looked upon as a rare reaction with little biological importance, more or less a chemical curiosity. The experiments described suggest that it is one of the most frequent and fundamental biological reactions, with close relations to the very nature of life. As has been pointed put, the main signs of life, motion, reflexes

and secretions are performed by structures brought about by the interaction of proteins with carbonyls. They are the result of the integrated action of many molecules, are thus linked to the entity, while the soluble proteins perform their function single-handed and can perform them equally well *in vitro* in molecular dispersion.

The experiments described have led to two unexpected findings, the catalysis of charge transfer between amines and dicarbonyls by sulfur, and oxygen. The SH group of glutathione catalyzes the charge transfer between amines and dicarbonyls, while molecular oxygen catalyzes the charge transfer between amines and unsaturated aldehydes. Oxygen thus has a dual role in biology: it acts as final electron acceptor in oxidation, and it catalyzes charge transfer. The failure of this latter function may be the immediate cause of the majority of human deaths. The immediate loss of consciousness, in anoxia, may be due to the cessation of the catalysis of charge transfer by oxygen. Brain has reserve energy in the form af ATP which could maintain activity for some time under anaerobic conditions. The immediate cessation of consciousness under anaerobic conditions suggest a more direct connection between brain function and oxygen supply.

With this information in hand, we can approach the problem of normal and pathological cell divison.

Cell division involves a complete rearrangement of the cellular interior. Such a rearrangement cannot take place in a solid structure. So, in order to be able to divide, the cell has to dismantle its structures, first of all its protein structure,which can be done by eliminating the carbonyls. Not only the protein structures are dismantled. The whole nucleus is broken down into chromosomes. Similarly, also mitochondria are dismounted forcing the cell to rely more on fermentation for its energy supply. Fermentation demands no structure.

The present research had its beginnings in the observation that tissue extracts contain a substance which inhibits cell division. The underlying substance was called "retine" and looked upon as a regulator. The experiments discussed indicate that cell division is not initiated by a specific regulator, but by a change in the whole physical state of the cell. "Retine" has a hand in this change of physical state, is a carbonyl.

If carbonyls dominate the physical state of the cell, and the cells contain active enzymes, as the glyoxalase, capable of inactivating the carbonyls, then it is evident that in the resting cell these carbonyls must be kept separated from the inactivating enzymes or else the enzymes must be present as proenzymes, being separated from their activator. In any case, such a situation demands a high degree of order, negative-entropy and cell division could be indicated by disorder, an increase in entropy, which allows enzyme or substrate, or proenzyme and its activator to meet, the result being the elimination of the carbonyls. Conversely, cell division could be arrested by allowing the cell to return to its orderly resting state by the production of carbonyls and a decrease in entropy.

If these considerations are correct, then the dividing cell should contain no carbonyls. The color of structures being due to carbonyls, the dividing cell

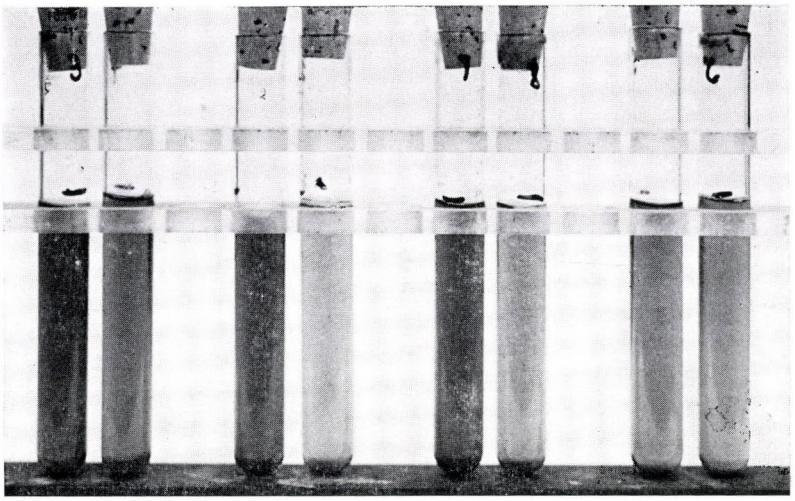

Fig. 8

should have no color. This point can be checked in the experiment by comparing the color of the regenerating rat liver with that of the resting one. As is generally known, 2/3 of a rats' liver, that is two of the three liver lobes can be cut out without killing the animal. The remaining lobe then grows within eight days to the size of the whole intact liver. On inspection, the regenerating liver is found to be definity lighter in color than the resting liver which was cut out. The color of the liver being partly due to blood, the blood had to be washed out before the final comparison was made. So, the extirpated lobes (representing the resting liver) and the regenerating lobes were blendored on the fourth day after the operation. The blendored ones were suspended in a great excess of distilled water, the proteins precipitated isoelectrically, and than resuspended in 10% laurylsulfate. As the Fig. 8 shows, the regenerating liver (right-hand side tubes) was definitely lighter colored as the resting one (right-hand side tubes). No complete discoloration could be expected because at the time of operation only part of the cells could have been dividing.

A semiliquid state had to be one of the main characteristics of *State I*. Dividing cells are semiliquid, as witnessed by the mushy nature of the mass filling healing wounds. Dividing cells show also other characteristics of *State I*, summed up on the left side of the Table 1. Liquids cannot differentiate und dividing cells are undifferentiated. In division cells depend for their energy more on fermentation, show low cohesion and have to be dielectric. We can thus sum up by saying that the cell, in division, has to return to the proliferative *State I*.

What lends prime medical import to these relations is the fact that after the cell has completed its division, it has to find its way back from the proliferative *State I* to the resting oxidative *State II*. If this road is blocked, the cells

have to stay in State 1 and go on proliferating senselessly, also when no proliferation is needed, and tumors are formed. This will be the case also when some noxious external agent disturbs the orderly *State II* and drives the cell back into *State I.* An andless variety of disturbances can do this. Any disturbance may do it which creates disorder and so doing brings the carbonyls and their inactivating enzymes together. Such a disturbance which elicits proliferation, may be a cut. If the disturbance is permanent, the proliferative *State I* has to become permanent too. One of the causes often made responsible for tumor formation, is temporary lack of oxygen. In absance of oxygen no carbonyls can be formed and so the protein has to lose its conductivity. The loss of conductivity interferes with oxidation, which depends on conductivity for tis "electron flow". This reduces the formation of carbonyls still further, driving the cell into a vicious circle which can be broken only by a timely introduction of carbonyls, which allow the cell to buold up its free radical protein complexes again.

Protein molecules being linked together to structures by carbonyls, we find the greatest part of carbonyls, in tissues, bound to protein structures. Only a small part of the carbonyls is free, in equilibrium with the bound ones, so that the elimination of the free carbonyls has to lead eventually to the release and inactivation of the bound ones. Conversely, the introduction of carbonyls has to lead to the increase in bound carbonyls and slow down proliferation. Hence, the carcinostatic growth-inhibitory action of tissue extracts containing carbonyls.

In the light of these studies cancerous state involves a return to *State I.* It has all the properties we ascribed to this State. Since, in the anaerobic *State I* there were no carbonyl acceptors, it follows that cancer should have no color. The difficulty of comparing the color of cancer with that of normal tissue is that it has a meaning only if we compare cancer with the analogous normal tissue. Such a comparison has been made possible to me by Dr. George Weber who kindly supplied me with a rapidly growing parenchymal rat, liver tumor (Morris Tumor 3924A). Such tumors and normal livers were blendored, their blood washed out, their proteins precipitated isoelectrically and resuspended with laurylsulphate. Fig. 9 (colour plate) shows such a suspension containing 20% liver (left) or tumor (right). The picture speaks for itself and demands no comment. The question is: could carbonyls actually have made this difference? This question is answered in Fig. 10 (colour plate) in which the right tubes contain the tumor suspension of Fig. 10, while the left one contains the same suspension incubated with 0.1 M croton aldehyde. It assumed the color of normal liver.

What has to be done, and done urgently, is to isolate, identify and synthetize the different carbonyls. I expect that these carbonyls will arrest cancer, and will do so without harming the patient. Till we have them in hand, we can experiment with the carbonyls which we have on our shelf, or which we synthetize. Since the natural carbonyls must be much more active than the artificial ones, I will touch only briefly upon the latter. Inhibitions of tumor growth of 50–100% have been achieved with them. The inhibition obtained was found to be inversely proportional to the size of the cancer inoculum. We usually inoculate our mice

with 10^7 cells, and obtain mostly a 50% inhibition. With an inoculum of 100 cells we got 80% inhibition. Extrapolated to 1 cell we can expect complete arrest of growth. This is important because cancer or a metastasis may start with one cell. This holds out the hope, that cancer can not only be cured, but can also be prevented, and eliminated altogether. Our substances acted also when given by mouth so that they can be administered without major inconvenience. Experiments on this line, performed with mice, showed that spontaneous cancer can be prevented to a great extent by the administration of dicarbonyls. A strong carcinostatic action of different carbonyls and carbonyl derivatives has been reported from different quarters.

We can expect different carbonyls to be associated with proteins performing different functions. This makes the chemistry of carbonyls into a wide and exciting new field if inquiry. The lack of these carbonyls may underlie different pathological conditions, holding out the hope that some of the carbonyls may become valuable items in the physician's armory. They all seem to have a small nmolecule promising a relatively easy synthesis.

What has led to the study of carbonyl was the subtlety and speed of biological reactions. These are most striking the in central nervous system, and so we can expect to find a specific carbonyl in the brain. This caused us to subject our whole concept to a test by attempting to isolate a specific carbonyl from brain. Fig. 11 (colour plate) shows dinitrophenylhydrazine of such a specific carbonyl in crystalline form. One of the unexpected qualities of this substance is that it tends to crystalize in the form of exceedingly thin, apparently endless threads (Fig. 12, colour plate). It is tempting to think that such threads, if associating with neurofilaments or tubules, make these latter conductant, transforming their system into a most complex conducting wiring. Judged by the complexity of its function, the brain, as a computer, must have an exceedingly complex wiring system. An exceedingly complex system of filaments is present in the brain cell in the form of its neurofilaments and tubules. Hitherto, science was unable to connect these with nervous functno they being built of proteins which were thought to be dielectrics. Should these threads be made conductant by the carbonyls then they could lend a physical basis to the function of the brain. It is an attractive idea to think that learning is a gradual development of orbital overlap between carbonyl acceptors and protein donors, while the elementary act of thinking actually could be the charge transfer, making the brain into a two-digit computer.

The Figures 4, 5, 8, 11 and 12 were quoted from my paper "Bioelectronics and Cancer" which appeared in Bioenergetics and were reproduced with the kind permission of the Plenum Publishing Company Limited.

Book Review

Transport Processes in Living Organisms by Z. Böszörményi, E. Cseh, G. Gárdos and P. Kertai. Akadémiai Kiadó, Budapest, 1972. 349 pages.

It may be considered a hopeless task for anyone to give a comprehensive account of biological transport processes on less than 350 pages. The only possible alternative the authors of the present volume could choose was to select some topics from the transport phenomena and have a monograph on these while deliberately omitting some other important subjects. This evidently means that we have here a competent treatment of some arbitrarily selected current topics in a volume where some others are entirely missing.

After a short introductory treatment of elementary transport processes by P. Kertai, the book has been divided into two parts. The first part written by Z. Böszörményi and E. Cseh deals with transport in plants including microorganisms. The second part includes transport in animal cells and organs: red blood cell transport is covered by G. Gárdos while the transport in the gastrointestinal tract, in the excretory organs and in the thyroid gland is described by P. Kertai. It becomes evident on examining the proportion of the individual parts within the volume that transport in plants is predominant on 128 pages, whereas red blood cells and animal organs occupy just 100 pages. Each chapter is supplemented by a list of references.

Three parts of the book deserve special mention in the reviewer's opinion. The chapter on transport processes in microorganisms is a review of the literature at the highest level. It is divided into sections on bacteria, yeasts, Neurospora and algae. This part can be used as an authoritative source of references. The chapter on transport across the red cell membrane is of different character. Its author has been deeply involved in the early pioneering work in this field: after describing the structure of the erythrocyte membrane, Dr. Gárdos gives a very detailed historical survey of the transport of monovalent cations, the development of the concepts of active transport as well as passive cation transport. The reader also finds a survey of anion permeability and of penetration of nonelectrolytes. The chapter is mainly based on the author's own investigations and is in fact an excellent summary of them. The third part deserving special attention is that on transport processes in the thyroid gland, since this is a rather neglected field. This section is again a review of literature: anyone who wishes to embark on this important field may have a good start by it.

The authors have apparently decided to omit intracellular transport in animal cells and tissues including transport in mitochondria and sarcoplasmic reticulum, as well as permeability and transport of the neural and muscle cell membrane and its physiological implications. The ionophorous compounds are not mentioned and therefore the carrier-mediated transport is not adequatly treated. The most serious shortcoming of the book is that the survey of the literature was closed in 1968 and due to a considerable delay in publication references after this date are missing. Probably for this special reason there is an appendix

containing a list of the most significant review papers between 1968 and 1971.

The book can be recommended for anyone who wants to have a comprehensive survey of literature in the field of microbiological, erythrocyte and thyroidal transport processes.

A. FONYÓ

Acta Histochemica, Suppl. XII. *Quantitative Histochemie der Proteine und Ribonukleinsäure, der Enzyme, Kohlenhydrate und Lipide.* Edited by D. Wittekind (Freiburg) and J. F. Jongkind (Amsterdam). VEB Gustav Fischer Verlag. Jena, 1972; 372 pages, 136 figures, 34 tables.

The German histochemists held their XIVth symposium joint with the Dutch society in Cologne in 1970. The theme in the title was dealt with in 32 lectures and discussed at 6 round-table meetings. The publication summarizes the material of 24 further free lectures partly joining the main theme.

I/a. Quantitative histochemistry of ribonucleoproteides. The quantitative measurement of RNP with ultraviolet photometric method and on the basis of their basic dye-binding was dealt with in connection with its structure and the effect of fixatives on fermentative extractability of RNP and the consecutive structural changes in electron microscopic structure. In the lecturers' opinion RNS synthesis can be well followed quantitatively with tritium-labelled nucleotides. At the same time they call attention to different errors in methodology which can lead to very delusive results and conclusions. I/b. Quantitative histochemistry of proteins. Because of their complex structure a lot of difficulties must be overcome in their determination. The generally accepted methods cannot be applied for in situ quantitative protein measurements. Similarly, the measurement of one component (amino acid) cannot provide information on the quantity of protein. Therefore the simultaneous determination in the same structure of several components with methods independent of each other is suggested with ultraviolet and fluorescence photometry, interferometry, measurement of dye-binding and autoradiographic method. A detailed description is given of the conclusion which can be drawn from the autoradiographic picture resulting from the simultaneous introduction of several labeiled amino acids, and theory of the mechanism of dyeing, its significance in practice, the determination of protein based on fluorometry and the perspectives of quantitative immunofluorescence.

II. Quantitative histochemistry of enzymes. In the opinion of the biochemists the methods of enzyme-cytochemistry give only qualitative or semi-quantitative results at the most.

Histochemists must therefore strive for joining the exact enzyme localization at cellular, subcellular levels and the data of quantitative enzyme chemistry. This is necessary also in order to compare cytochemical results with findings obtained by differential centrifugation. The theme was mainly reported by Dutch colleagues, but one can also read lectures of Hungarian authors. Beside cytophotometry chiefly methods of fluorometry are used in determining enzyme activity, though the lead atom absorption photometric measurement was also reported in connection with the alkaline phosphatase reaction of Gömöri. The effect of fixation and denaturation on enzyme activity was dealt with and attempts were made to express the quantitative cytochemical results in biochemical terms. It is regrettable that only a few methods are known at present for in situ demonstration of isoenzymes of increasingly greater importance. Another meeting dealt separately with ultramicrochemical enzyme examinations with the fluorometric determination of enzyme activity (dehydrogenase, ATPase) of fixed cells isolated by means of the microdissection method.

III. Quantitative histochemistry of polysaccharides. The structure of homo- and heteropolysaccharides and the structural criteria determining the basophilia and metachromasia of the latter are dealt with.The microchemical analysis of glycose amine glycanes is discussed in the light of different methods of separation, including section chromatography, electrophoresis, and the quantitative polarization optical analysis

of mucopolysaccharides. The dynamics of epithelial and mesenchymal mucus formation was discussed on the basis of radiochemical examinations with electronmicroscopic autoradiography.

IV. Quantitative histochemistry of fats. Beside the testing of the lipid solving effects of different aqueous (formol) fixatives, the solubility of lipid fractions during embedding after osmium treatment was also dealt with. The lecture concerning the histochromatography of lipid raised great interest, just as did the observation concerning the lipid analysis of biopsic samples, which is important also from a diagnostic point of view.

V. Among the free lectures, beside enzyme chemical examinations of wide spectrum, it is worth mentioning the report dealing with the quantitative histochemistry of DNS, the alteration of tissular enzymes following enzyme induction, the quantitative analysis of the polysaccharide layer (glycocalix) on the surface of the membrane as well as the X-ray microanalysis of intracellular electrolytes. The communications are completed by numerous figures and pictures. Beside reference to the literature, we can find a detailed discussion. The conclusion of the symposium is that the histochemists have to overcome a lot of difficulties in order to obtain exact quantitative data with their methods.

K. JOBST

Biocybernetics. Volume IV. Edited by H. Drischel and P. Dettmar. VEB Gustav Fischer Verlag. Jena. 1972; 320. pp.

This book is the fourth one in the series of Proceedings of International Symposia on Biocybernetics. This latest volume comprises the lectures delivered on the IIIrd International Symposium on Biocybernetics which was a Satellite Symposium of the XXVth International Congress of Physiological Sciences held under the chairmanship of W. D. Keidel and H. Drischel at Leipzig, German Democratic Republic, August 3–7, 1971. The book consists of the presented papers dealing with five different topics, the common bond of which is the "Modelling biological systems and mathematical analysis of biological processes".

The first and longest section of this volume has been devoted to the basic, general and theoretical aspects of biocybernetics. The main emphasis in this section is given to modelling activities of different neuronal nets and the processes of pattern recognition. Some of the results discussed in these articles have been connected to physiological experiments, however, some others would yet need this kind of connection because without this the results have significance only from the point of view of pure mathematics.

The second section contains the papers concerning the experiments performed on visual system. These articles can be divided in two groups. One of them deals with the studies of the excitatory processes taking place in the retina, and the second group presents results of investigations of the optomotor system.

The third section of the book comprises the presented papers concerning the control processes connected with the different aspects of muscular activity. The articles in this part describe the results of studies on the structural and functional basis of the regulation of muscle contraction, on the non-linear properties of the motor system and on the activity of muscle receptors. Despite the remarkable range of material covered in this section, some shortcomings can be also found in the details of some articles. In an article, the expression "advantage" is used many times. This expression would be completely right for a technical system, but it is not proper for a biological one. Contrary to the statement in this article, to control the position of the head in space is not the primary function of the motor system. In another article, it could have been mentioned that the stiffness of muscle can be produced not only by the reflex activity but by crystallization of muscle proteins owing to passive stretch of the muscle. In another place there is an unusual data of 50–60 msec in connection with the transformation of the muscle electrical activity into a mechanical one.

The next two sections of this volume contain papers dealing with the biocybernetical viewpoints of the sensomotor systems, especially eye and head pursuit movement,

and of the autonomic homeostatic systems and the cardiovascular system in particular.

Beside the five sections mentioned above, there is a summary of the round-table discussion "Modelling Biological Systems and Mathematical Analysis of Biological Processes" devoted to discussing the basic problems involved in building of models and the possible future development of biocybernetics.

Since the publication of N. Wiener's book "Cybernetics" in 1948 many biologists have been interested very much in this rapidly developing field. It is generally accepted that the cybernetical methods could be very useful for understanding the processes taking place in different biological systems. However, the lectures delivered at this congress have also shown that the first and by far too optimistic phase of biocybernetics has been overcome. Many authors came to the conclusion that the simulations of even elementary functions should still wait for further developments and the right methods had not yet been found. This congress called attention to another problem, too. One of the editors of this book, H. Drischel, emphasized the point that about one third of all contributions to the symposium had proved to be "pseudocybernetics", nevertheless, there is hope that this kind of works will be relegated.

G. Biró

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Physical Evidence for the Linkage of 16 *S* and 23 *S* rRNA Cistrons in *Escherichia coli*

(Short Communication)

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(Received April 18, 1973)

Recently we described a method for the purification of *Salmonella typhimurium* DNA fragments coding for ribosomal RNA (Udvardy, Venetianer, 1971). This purification made possible the direct determination of the contiguity of 16 *S* and 23 *S* rRNA cistrons in *E. coli* by an approach in principle similar to that used by Colli and Oishi (1969) in the case of *Bacillus subtilis*.

[^{32}P]-labelled total DNA of *E. coli* MRE 600 was disintegrated by controlled ultrasonic treatment to fragments with defined average single-stranded molecular weight. The DNA fragments were denatured with NaOH and the complementary strands of rDNA were separated by chromatography on methylated albumin-kieselguhr (MAK) columns (Udvardy, Venetianer, 1973). The fractions containing the bulk of the transcribing strands of rDNA were hybridized with unlabelled 23 *S* rRNA from the same strain. The rRNA – DNA hybrids were separated from the rest of the DNA by chromatography on deoxycholate-treated BD-cellulose (Udvardy, Venetianer, 1971). The hybrids were then treated with NaOH to remove the rRNA, and the DNA was fixed onto nitrocellulose filters (Sartorius SM 11306, 50 mm format). The filters were cut into small pieces, each carrying approximately

Table 1

Hybridization of 16 S and 23 S rRNA

16 *S* and 23 *S* rRNA were purified by phenol extraction of ribosomes and repeated chromatography on MAK columns. Their purity was checked by polyacrylamide gel electrophoresis. DNA was fragmented by 15 sec and 30 sec ultrasonic treatment with an MSE 100 W disintegrator in 30 ml batches (50 μg/ml). The average molecular weights were determined in alkaline sucrose gradients using linearized $\phi \times 174$ DNA as reference standard. All hybridizations were done in duplicate, and the values were normalized with respect to the DNA content of the filters. Blank values (filters without DNA) were subtracted. All other methods have been described in detail elsewhere (Udvardy, Venetianer, 1971, 1973)

[^{32}P]-rDNA	[^{3}H]-rRNA	Hybridized cts/min [^{3}H] / μg [^{32}P]-rDNA	Ratio of hybr. 16 *S*/23 *S* rRNA
1.25×10^6	16 *S*	480	0.46
	23 *S*	1042	
0.7×10^6	16 *S*	1305	0.29
	23 *S*	4530	

0.1 μg DNA and they were hybridized separately with purified [^{3}H]-labelled 16 *S* or 23 *S* rRNA. This experiment was carried out with DNA preparations fragmented to two different sizes. In one case the average molecular weight of the fragments (after the purification procedure) was larger than that of the 23 *S* rRNA but smaller than that of the 23 *S* and 16 *S* rRNA together. In the second case the fragments were smaller than the 23 *S* rRNA.

If the 16 *S* and 23 *S* rRNA cistrons were unlinked, one would not expect any hybridization with 16 *S* rRNA in either experiment. If the two cistrons were contiguous, the 16 *S* rRNA would hybridize to the fragments purified by hybridization with 23 *S* rRNA. With the larger fragments this hybridization would be close to the maximal 1 : 2 ratio (16 *S*/23 *S*), but it would be much lower with the smaller fragments. Table 1 summarizes our experimental data which bear out the latter prediction.

These results are in agreement with kinetic and electron microscopic observations, also supporting the notion that the 16 *S* and 23 *S* rRNA cistrons are contiguous on the *E. coli* chromosome (Doolittle, Pace, 1971; Pato, von Meyenburg, 1970; Kossman et al., 1971; Miller et al., 1970).

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The Heterogeneity of Informofer III. Electronmicroscopic Studies on Various Types of Cell Nuclear Particles Containing dRNA

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(Received November 23, 1972)

dRNA-containing particles of different properties can be obtained from the nuclear fraction of rat liver if the extracting buffer contains 0.3 M NaCl. The submicroscopic properties of the ribonucleoproteid particles thus prepared were studied and compared to the particles extracted in the usual way with 0.1 M NaCl. The dRNA-containing particles isolated with 0.3 M NaCl gave a more heterogeneous population as revealed by both negative contrast and platinum–palladium shadowed electron micrographs, than did the particles isolated with 0.1 M NaCl. The two types of particles also displayed heterogeneity in CsCl equilibrium density gradient. All these data suggest that the heterogeneity of these particles is manifest at both the biochemical and the submicroscopic levels.

Introduction

The cell nuclear particles containing dRNA, in which RNA is bound to a globular protein component, the informofer, have well defined submicroscopic properties in addition to their biochemical characteristics (Samarina et al., 1967; 1967a). These 30 *S* particles, which are extracted from the nucleus with buffer containing 0.1 M NaCl, are 180 to 200 Å in size and exhibit marked homogeneity on electron micrographs.

If extraction with buffer containing 0.1 M NaCl is continued with a buffer containing 0.3 M NaCl, a rapidly labelling dRNA fraction can be extracted, which under physiological conditions does not leave the nucleus (Drews, 1969). This dRNA fraction is complexed to such informofers the protein composition of which differs from that of previously described informofers, insofar as the protein composition is simpler and the susceptibility of particles to RNAse is increased (Molnár, Juhász, 1972).

In the present work we undertook the electron microscopic analysis of nuclear particles containing dRNA and extracted with buffer 0.3 M in NaCl. We sought the answer to the question, what submicroscopic features these particles display, in addition to their biochemical characterictics, in comparison with nuclear particles extracted in the usual way with 0.1 M NaCl. Our observations indicate that the particles obtained with 0.3 M NaCl are heterogeneous not only biochemically but also in their submicroscopic pattern.

Materials and methods

In the experiments CFY white rats of both sexes were used. To the animals 20 μCi [^{14}C]-orotate was administered intraperitoneally 30 minutes before killing. In part of the experiments double labelling was applied, in which case $Na_2H^{32}PO_4$ (1.0 μCi per animal) was given 2 hours before killing and 20 μCi [^{14}C]-orotate 20 minutes preceding killing.

The nuclear fraction was prepared from the animals' liver by the modified Chauveau method (Samarina et al., 1967), from which the particles containing dRNA were obtained as follows. The nuclear fraction was washed with STM I (0.1 M NaCl, 0.001 M $MgCl_2$, 0.01 M Tris.HCl, pH 7.2), then twice extracted with STM II (the same as STM I but the pH is 7.8 – 8.0) as described earlier (Samarina et al., 1967). The extracts thus obtained were pooled; this fraction will be referred to as "0.1 M extract." Then the nuclear fraction was extracted twice with STM III buffer, which is the same as STM II, but contains 0.3 M instead of 0.1 M NaCl (Molnár, Juhász, 1972). The two extracts obtained were pooled and will be referred to as "0.3 M extract".

The above two types of extract were centrifuged in a 15 – 30% (w/w) linear sucrose gradient in the 3 × 5 ml SW rotor of a Janetzki VAC 601 ultracentrifuge at 38 000 r.p.m. and 4°C. Fractions of 3 drops were collected and tested for light absorbance at 260 nm and radioactivity.

The nuclear ribonucleoproteid containing dRNA was purified by gel-filtration in those cases when the particles were to be sedimented in a CsCl equilibrium density gradient (Molnár, Komáromy, 1972a). The bouyant density of ribonucleoproteid fixed with formaldehyde was determined as described earlier (Samarina et al., 1967a).

The material purified by gel-filtration, fixed with formaldehyde and dialyzed was mixed with CsCl solutions (1.25 to 1.55 g per ml) containing 0.005 M phosphate buffer, pH 7.4, and 2% formaldehyde. The samples were sedimented in the 8 × 10 ml rotor of a Janetzki VAC 60 ultracentrifuge at 42 000 r.p.m. for 18 hours at 10°C. Fractions of 5 drops were collected and the bouyant density was measured in every fifth fraction of the gradient by means of a refractometer.

For the determination of acid-insoluble radioactivity of the fractions, they were washed onto cellulose nitrate filters (Synpor 2) and counted with an end-window GM tube (^{32}P) or with a Gamma Scintillator (^{14}C). In the latter case we used an apolar scintillator liquid (4 g PPO, 100 mg POPOP in 1000 ml toluene).

For the examination of the submicroscopic properties of particles obtained by extraction with 0.3 M NaCl-buffer (in the following "0.3 M particles"), and for the comparison of these "0.3 M particles" with those obtained by extraction with 0.1 M NaCl-buffer (in the following "0.1 M particles"), the gradient samples containing the particles were prepared for electron microscopic analysis.

The sucrose gradient samples, which contained the 30 *S* particles, were fixed with 20% neutralized formaldehyde solution. The final concentration of formaldehyde was 2%. The fixed material was dialyzed through a 2% parlodium membrane against STM buffer pH 7.2 (which contained 0.01 M NaCl). Part of the

dialyzed material was mounted on parlodium carrier membrane electron microscopic grids and stained with aqueous 0.5% uranyl acetate solution.

Another aliquot of the material containing 30 *S* particles was prepared according to the basic protein-film technique (Láng, Mitani, 1970). The protein-film layer was taken up onto a formvar membrane fortified with carbon membrane.

The specimens thus prepared were shadowed with platinum–palladium vapor at an angle of 6°20′ in a Zeiss Jena HBA 120/2 type instrument (Bradley, 1965).

The electron microscopic preparations obtained by the above two procedures were analyzed in a TESLA BS 513 type electron microscope at 70 kV voltage, the preparations shadowed with platinum–palladium at 10 000-fold magnification, whereas the negative contrast preparations at 35 000-fold basic magnification.

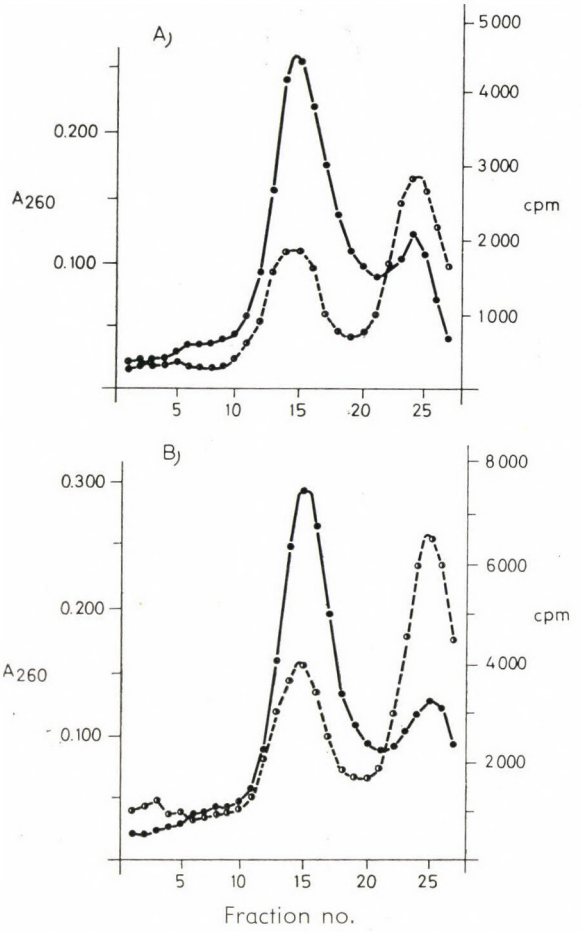

Fig. 1. Ultracentrifugation of "0.1 M" (A) and "0.3 M" (B) extracts in linear sucrose gradient. 0.3 ml nuclear extract was layered on top of 4.8 ml 15–30% (w/w) sucrose gradient. Centrifugation was carried out in the 3×5 ml rotor of a Janetzki VAC 601 ultracentrifuge for 4 hours at 38 000 r.p.m. and 2°C. Three-drop fractions were collected. ◑—◑, A_{260}; ●—●, [^{14}C]-radioactivity, c.p.m.

Results and discussion

The "0.1 M extract" and "0.3 M extract" when ultracentrifuged in a 15 to 30% (w/w) sucrose gradient gave a radioactivity peak that originated from [^{14}C]-orotate in the 15th fraction (Fig. 1). This pattern is expected for the occurrence of 30 *S* particles if the isolation is performed without RNAse inhibitor (Samarina et al., 1968; Molnár, Juhász, 1972).

The zone of 30 *S* particles, which contain the dRNA complexed with informofers, when subjected to electron microscopic analysis after suitable preparation gave the following submicroscopic picture. The "0.3 M particles" exhibited marked heterogeneity as compared to the "0.1 M particles" both on the negative contrast micrographs and on the pictures obtained with shadowing with platinum–palladium vapor. The diameter of "0.1 M particles" is about 190 – 200 Å as measured on the negative contrast pictures (Fig. 2A). The same value was arrived at in measurements on preparations shadowed with platinum–palladium vapor (Fig. 2B). Samarina et al. (1967; 1968) have obtained similar results.

In contrast, the average diameter was found to be 210 – 220 Å for "0.3 M particles" both on the basis of negative contrast (Fig. 3A) and platinum–palladium shadowed pictures (Fig. 3B). The shadow dimensions of "0.3 M particles" measured on metal vapor-treated preparations fall within the limit values of 134 and 290 Å whereas those of "0.1 M particles" varied between 140 and 250 Å (Fig. 4). The latter values are in agreement with the data of Samarina et al. (1967; 1967a; 1968). As seen from the above measurements, the limit values are about the same for the two types of particles, but there is appreciable difference in the distribution pattern of diameter values within the observed range for the two particle types: the "0.3 M particles" have a more heterogeneous population than "0.1 M particles."

There was no significant difference between the diameters of "0.3 M particles" and "0.1 M particles" as evaluated statistically by Student's *t*-test ($p > 0.10$).

This result derived from the statistical determination of dimensions is in accord with the pattern that emerged from sedimentation in 15 – 30% (w/w) sucrose gradients, as far as both the "0.1 M particles" and "0.3 M particles" appeared in the same fraction after ultracentrifugation. The problem remains, however, what is the explanation of the greater heterogeneity observed between the extremes in the case of "0.3 M particles." Our data obtained with CsCl equilibrium density gradient centrifugation (Fig. 5) offer an interpretation. It is apparent from the pattern that part of the "0.3 M particles" appears in the zone of 1.37 gcm^{-3}, whereas another part is found in the layer of density 1.39 gcm^{-3}. The [^{14}C]- and [^{32}P]-radioactivity curves are in full agreement with this picture. It should be noted, however, that this heterogeneity is consistently found only if the extraction of particles is performed in the absence of RNAse inhibitor.

It follows from the foregoing that the "0.3 M particles" represent a more heterogeneous population than the 30 *S* nuclear particles extracted in the usual way. Another explanation of these observations might reside in the finding of Molnár and Juhász (1972), according to which "0.3 M particles" lose part of their RNA content during preparation and the ratio of protein components of

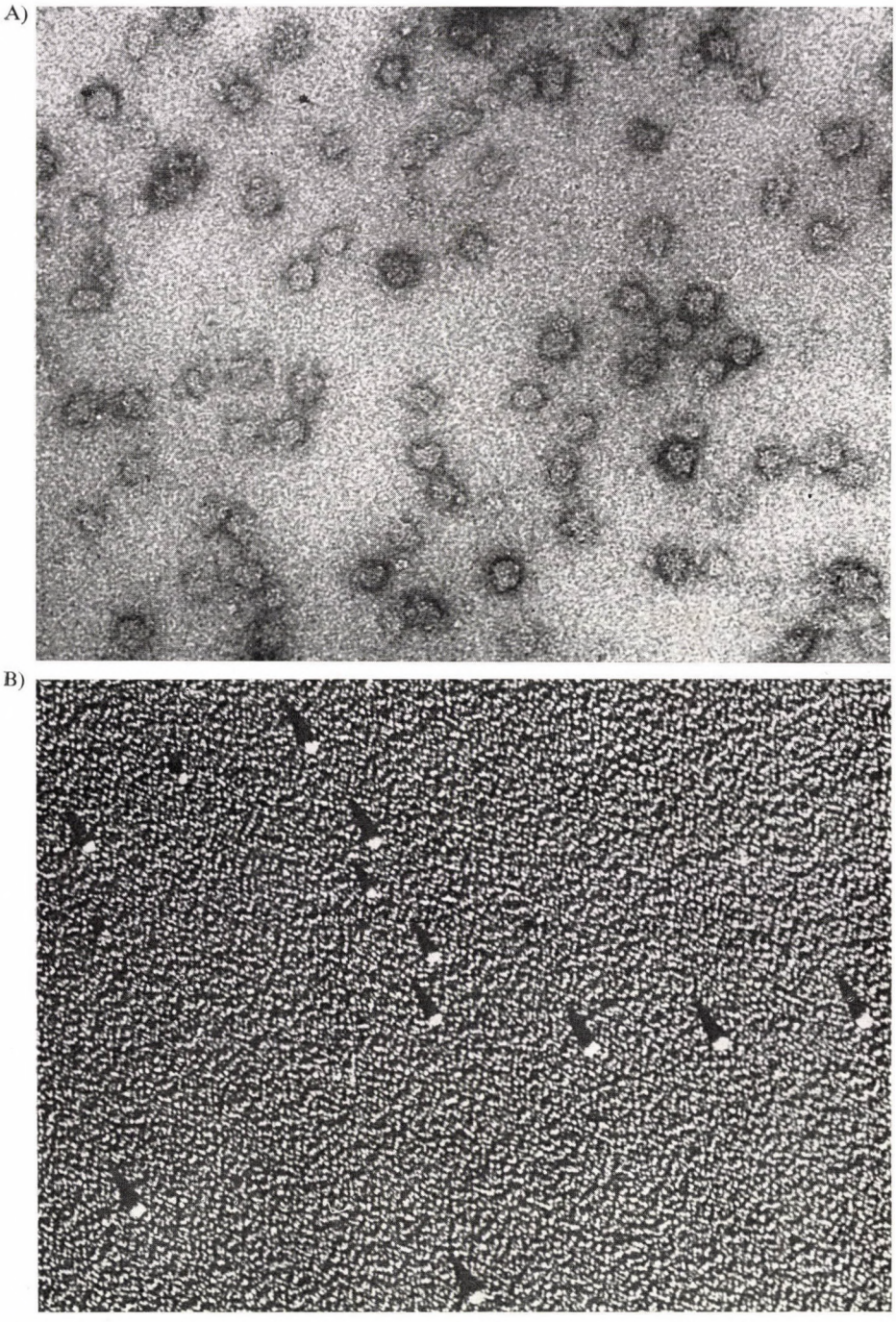

Fig. 2. Electron micrograph of dRNA-containing 30 *S* particles extracted from the nucleus with buffer containing 0.1 M NaCl. A) Negative contrasting with 0.5% uranyl acetate. Magnification 165 000×. B) Platinum–palladium shadowing. Magnification 40 000×

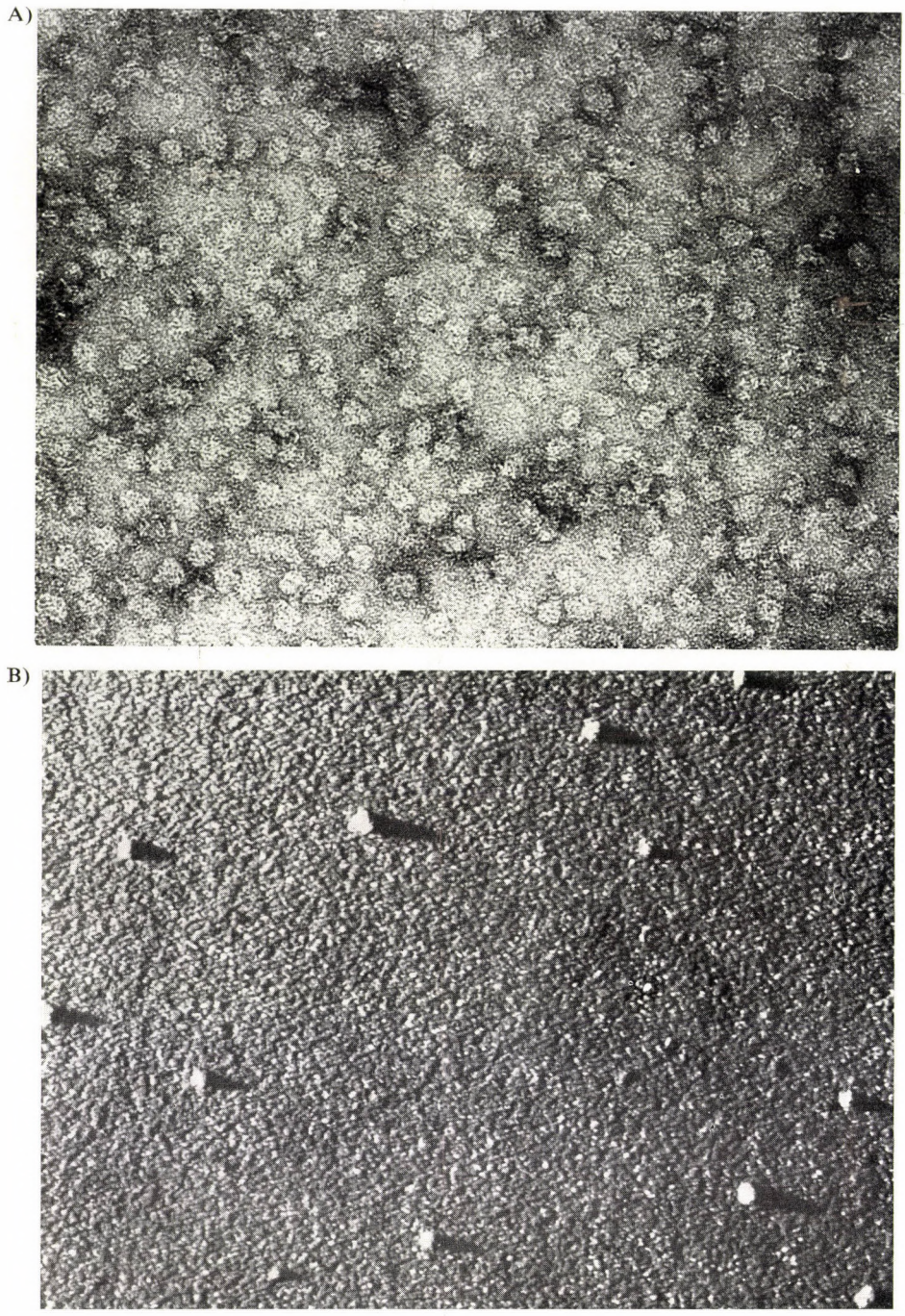

Fig. 3. Electron micrograph of dRNA-containing 30 *S* particles extracted from the nucleus with buffer containing 0.3 M NaCl. A) Negative contrast technique, 0.5% uranyl acetate. Magnification 165 000×. B) Platinum–palladium shadowing. Magnification 40 000×

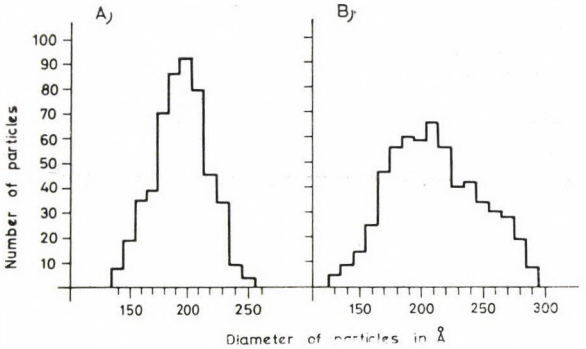

Fig. 4. Statistical distribution of diameter values of dRNA-containing 30 *S* particles extracted from the nucleus with buffe.s containing 0.1 M NaCl (A) and 0.3 M NaCl (B), as measured on the electron micrograph of their platinum–palladium shadowed preparations

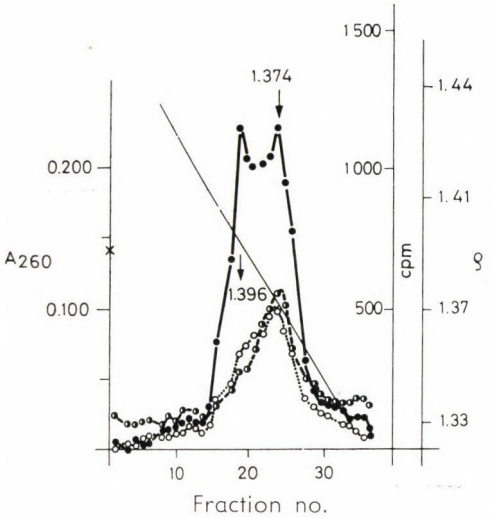

Fig. 5. Equilibrium density gradient centrifugation in CsCl of "0.3 M particles" purified by gel-filtration. Ultracentrifugation was carried out in the 8×10 ml rotor of a Janetzki VAC 60 ultracentrifuge for 18 hours at 42 000 r.p.m. and 10°C. Five-drop fractions were collected. ◑—◑, A_{260}, ●—●, [^{14}C]-radioactivity, c.p.m. ○—○, [^{32}P]-radioactivity, c.p.m.

"0.3 M particles" differs from that of "0.1 M particles": component B is markedly increased relating to components A and C. This fact might influence the behaviour observed during the preparative work, which finally may result in a more heterogeneous appearance. This seems to be supported by our observations according to which the submicroscopic features of dRNA-free informofers in which the ratio of protein components is the same as in the dRNA-containing informofer, the 30 *S* particles, do not differ from those of dRNA-containing informofers (Lukanidin et al., 1972). Heterogeneity might also be influenced by the fact that the extraction method does not permit the complete separation of the two types of dRNA. The "0.1 M particles" also comprise dRNA that is degraded in the nucleus (Samarina et al., 1968). From the CsCl equilibrium density gradient ultracentri-

fugation pattern it can be assumed that the "0.1 M particles" are also found in the "0.3 M extract" (Molnár, Juhász, 1972).

Our results indicate that the dRNA-containing informofers extracted with 0.3 M NaCl differ in their submicroscopic properties from those dRNA-containing particles that are extracted in the usual way with 0.1 M NaCl. The differences involve both size and shape. These findings are in accord with biochemical data: biochemical heterogeneity is accompanied by submicroscopic heterogeneity.

Thanks are due to Dr P. Juhász for his valuable advices and to Mrs F. Studinger for devoted technical assistance.

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Adenosine Phosphate Concentrations and Carbohydrate Consumption in the Tissues of *Anodonta cygnea* L. (Mollusca, Pelecypoda) under Normal and Anoxic Conditions

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1. During the first 6 days of complete anoxia, 52–94% of normal ATP-content was measured in the total animal, while the total adenylate pool decreased to 53% in average. The ATP concentration was only 11% of the normal on the 7th day of anoxia when the animals began to die.

2. In contrast to the expectations, the ATP/ADP and ATP/AMP ratios increased during the first 6 days of anoxia and decreased deeply below the normal level only later.

3. The value of "energy charge" was 0.43 in normal animals, it increased during anoxia reaching its maximum (0.58) by the 3rd day, then decreased again and became lower (0.28) than the normal only on the 7th day.

4. The glycogen content of the tissues decreased during anoxia to the same extent as the animals were kept under the same conditions but supplied with oxygen.

5. It seems likely that the energy supply is maintained during anoxia by a mechanism called "anoxic endogenous oxidation," which allows an oxidative energy production at the expense of an intrinsic electron acceptor for a relatively long time.

Introduction

It has been shown that in the tissues of *Mytilus galloprovincialis*, maximally 60% of the normal ATP-content is present during prolonged anoxia (Zs.-Nagy, Ermini, 1972b). The possibility has been outlined in several papers that the yellow pigment granules (cytosomes) correspond to the energy-yielding cell organelles during anoxia, which are always present in tissues of molluscs displaying a high degree of anoxic tolerance (Zs.-Nagy, 1971a, 1971b, 1973a; Zs.-Nagy, Kerpel-Fronius, 1970; Zs.-Nagy, Borovyagin, 1972; Zs.-Nagy, Ermini, 1972a). The possible mechanism of cytosomal energy production has been called "anoxic endogenous oxidation" (Zs.-Nagy, Ermini, 1972b), the essential point of which is that some of the components of cytosomes substitute for the electron acceptor function of molecular oxygen under anoxic conditions. It has been proved experimentally that the ganglia of *Anodonta cygnea* contain an ethanol soluble pigment substance, which is able to maintain a redox potential level higher than that of cytochrome oxidase in the tissue even during complete anoxia (Zs.-Nagy, 1971b). Thus the realization of the mechanism of "anoxic endogenous oxidation" is theoretically possible. The present work was aimed at the detailed investigation of anoxic energetical processes of molluscs in a species showing an even better anoxic tolerance than that of *Mytilus* (Brand, 1946).

Materials and methods

Adult specimens of *Anodonta cygnea* of 12– 16 cm body length, were used. Groups of three animals were placed in Balaton-water amounting to a volume 8 times the total weight of the animals. The temperature varied between 14 and 16°C when adenosine phosphates were measured and was 15 ± 0.5°C during the analysis of carbohydrates. The animals were abundantly supplied with air for 24 hr. During this period they fed on the plankton of water and got accustomed to the constant temperature. From the end of this period the animals starved. At this point some of the groups were brought into anoxia by covering the water surface with a paraffin oil layer of 1–2 cm as a consequence of which the oxygen content of water was gradually used up. According to polarographic measurements, the water did not contain detectable amounts of oxygen by the 17th hr after covering the water surface. Other groups of animals were abundantly supplied with air even further. The following groups were investigated:

a) Adenosine phosphate controls: 3 normally respiring and feeding animals.

b) Anoxic adenosine phosphate groups: Three animals were examined daily up to the 7th day after covering the water surface.

c) Carbohydrate consumption under normal respiration: The total carbohydrate and glycogen contents of 3 animals were measured daily up to the 7th day.

d) Carbohydrate consumption in anoxia: The same as under c) but these animals were kept in anoxia. The period of starvation of the animals compared in groups c) and d) was always identical.

Measurements of adenosine phosphates

The same method was used as in the case of *Mytilus galloprovincialis* (Zs.-Nagy, Ermini, 1972b). The essential points of the method are the following: the total body of the animal without shells is homogenized in perchloric acid, then after neutralization and centrifugation the supernatant is put on an ion exchange column, which binds all the adenosine phosphates. Fractions containing in turn AMP, ADP and ATP can be obtained by gradient elution. The concentrations of the adenosine phosphates in the fractions were measured spectrophotometrically at 260 nm. As regards the details we refer to the paper of Deutsch and Nilsson (1953).

Measurement of carbohydrates

The determination of the total carbohydrate content was carried out by the anthrone method after homogenization of the total body in 1.0 N H_2SO_4 and hydrolysis in boiling water bath for 2 hr (Colowick, Kaplan, 1957). The non-hydrolysed tissue residues were sedimented by centrifugation after neutralization and the carbohydrates were measured by calibration to a standard glucose solution.

The measurement of glycogen concentration was also carried out by the anthrone method but after hydrolysis in hot 30% KOH. In the preliminary experiments glycogen was precipitated (Colowick, Kaplan, 1957). However, since the

glycogen content was always well above 1% in the tissues, we used the method of Seifter et al. (1950), which allowed us to determine such high glycogen concentrations in the presence of protein. The results obtained in the same homogenate by the two methods agreed well, therefore later the precipitation of glycogen was omitted. The spectrophotometric measurement was performed at 620 nm on the basis of a standard glucose calibration curve. The values are expressed in glycogen content calculated by the aid of a suitable correction factor (0.9, Ploetz, 1955).

Results

1. Adenosine phosphate concentrations

The results are summarized in Table 1. During the first 6 days of anoxia 52–94% of the normal ATP-content was found in the animals, whereas by the 7th day it decreased to 11%. That day the animals started to die. At 14–16°C generally all animals live by the 7th day but none of them survives the 10th day.

Table 1

Concentrations of adenosine phosphates and the calculated parameters of the adenylate pool in normal and anoxic animals
The concentrations are given in nmoles/g wet weight

		Normal animals	1st	2nd	3rd	4th	5th	6th	7th
			day of anoxia						
AMP	Mean	117	61	38	29	44	51	96	56
	Range	74–159	24–87	21–52	23–38	39–49	35–67	35–128	34–82
ADP	Mean	106	55	49	46	48	44	68	47
	Range	87–140	34–88	34–66	37–60	37–56	39–53	52–90	19–94
ATP	Mean	73	38	60	49	59	43	69	8
	Range	56–85	26–53	45–76	43–54	41–85	34–50	64–77	2–14
Total adenosine phosphates in average		296	154	147	124	151	138	233	111
ATP as a per cent of normal level in average		100	52	82	67	81	59	94	11
$\frac{ATP}{ADP}$		0.69	0.69	1.22	1.07	1.23	0.98	1.01	0.17
$\frac{ATP}{AMP}$		0.62	0.62	1.58	1.69	1.34	0.84	0.71	0.14
$\frac{ATP + 1/2\ ADP}{ATP + ADP\ AMP}$		0.43	0.43	0.57	0.58	0.55	0.47	0.44	0.28

Apart from the range values, each figure represents the average of 3 measurements in different animals.

Table 1 includes the ATP/ADP ratios. This parameter was normal on the 1st day of anoxia, and it increased from the 2nd day. Its maximum was reached by the 4th day, when it was about 77% higher than the normal. On the 6th day it decreased again but was still above the normal. By the 7th day it decreased to a very low value. Similar changes appeared in the ATP/AMP ratio (Table 1).

The "energy charge" parameter (Atkinson, Walton, 1967) was normal on the 1st day of anoxia, then it increased, reaching its maximum by the 3rd day. In fact, it was near this value from the 2nd till the 4th day. From the 5th day it gradually decreased but became lower than the normal only by the 7th day.

2. *Carbohydrate consumption*

The glucose equivalents of the total carbohydrate and glycogen contents measured in the total body of animals showed only a very small, statistically insignificant difference. This indicates that the great majority of the stored carbohydrates is present in the form of glycogen in the tissues of *Anodonta*. Considering the glucose concentration of hemolymph of several bivalves (7–65 mg%, Goddard, Martin, 1966), as well as the fact that the hemolymph amounts to about 50% of the body weight (Martin et al., 1958), one can expect a maximal amount of glucose dissolved in the hemolymph of *Anodonta* of about 30–35 mg per 100 g tissue. Since the glycogen content is very high in wet tissue, more than 4%, it is clear that the dissolved glucose mentioned above amounts to only less than 1% of the total carbohydrate content. Consequently, it seems quite sufficient to follow the changes of glycogen concentration in order to monitor carbohydrate consumption during starvation. It should be noted that the study of starving animals is advantageous for our purposes from several points of view: i) it eliminates the source of error that the carbohydrate content of the food cannot be precisely determined; ii) the transformation of carbohydrates into lipids is unlikely to proceed during starvation, i.e., the carbohydrate consumption observed can be regarded as the real amount of sugar used up for energy production. According to our preliminary measurements the glycogen content is very high, therefore one can suppose that the endogenous carbohydrates are sufficient for prolonged experiments up to 7–8 days. On the basis of the foregoing, we treat in detail only the glycogen measurements.

The glycogen content is about 4–5% of the wet weight in the normal animals (Table 2) during May and June. However, the values vary in a wide range between 2 and 7%, sometimes even between 1 and 2%. In oyster 4.38% glycogen-content was reported (Mitchell, 1915). Recently, 10–35% of dry weight was found in *Mytilus edulis* (De Zwaan, Zandee, 1972a), which is in good agreement with our data, since the water content of molluscan tissues is about 80% (Prosser, Brown, 1964). Since because of the wide statistical scattering of data the glycogen concentrations cannot be compared by simple averaging, we used the regression analysis (Fig. 1). The regression coefficient proved to be -156.98 ± 118.48 mg/100 g wet tissue/day. The wide scattering is also reflected in the high standard deviation of this coefficient.

Table 2

Glycogen content of animals under normal and anoxic conditions, at 15.0±0.5°C

Days	Glycogen content, mg/100 g	
	Normal	Anoxia
1	5191.0	4012.5
1	1515.0	4713.3
1	4080.6	4326.9
1	3409.2	6834.8
1	4383.9	3589.6
1	5814.9	—
2	4585.5	7138.8
2	6323.4	2176.2
2	2916.9	6935.4
2	—	4608.1
2	—	5238.0
3	—	3827.7
3	—	6129.9
3	—	6387.3
4	3150.0	6078.6
4	5699.9	1631.2
4	4221.9	4944.6
5	3750.0	4137.3
5	6283.8	4449.9
5	5111.1	5826.6
6	6780.9	2801.7
6	4725.0	7641.9
6	3049.7	5549.4
7	2814.3	3612.0
7	1901.2	4243.3
7	1482.8	4190.4
8	3904.0	3801.6
8	5225.2	2814.1
8	5224.5	5141.1

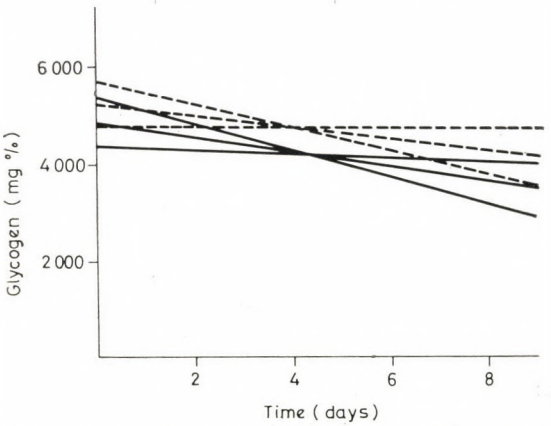

Fig. 1. Normal and anoxic glycogen consumption of *Anodonta cygnea* L. calculated on the basis of the regression coefficients ± s.d.

In 8-day-anoxia the regression coefficient was -119.59 ± 120.36 mg/100 g wet tissue/day. This is somewhat lower than the normal value, but the difference shows only a $P > 0.50$ value of significance, i.e., the normal and anoxic glycogen consumptions are practically identical. One can calculate that if the anoxic carbohydrate consumption of the animals were even only 2–3 times as high as the normal, the difference of the regression coefficients would be statistically significant despite of the wide scattering of values.

The results suggest that the carbohydrate consumption of these animals does not increase significantly under anoxic conditions as compared to normal respiratory circumstances . At least it is certainly not higher than twice the normal.

Discussion

According to our results, the tissues of *Anodonta cygnea* possess an appreciable ATP content during the first 6 days of anoxia, corresponding to 52–94% of the normal level. At the same time the total adenylate pool decreases to 53% in average. The decrease is specially pronounced in the AMP fraction during the first 5 days of anoxia, which can be connected with deamination processes.

The ATP/ADP ratio is an indicator of the phosphorylating potential of tissues (Klingenberg, Pfaff, 1968), as well as of the energetic state of the cells (Newsholme, Gevers, 1967). The values are known for different organs of mammals: in the liver of adult rats it is 3.3, in that of newborns 7.8–8.5 (Ballard, 1970; Philippidis, Ballard, 1970); in the brown adipose tissue of hamster 4.6 (Williamson, 1970); in isolated respiring rat kidney 2.4 (Hems, Gaja, 1972); in rat brain in vivo 5.5 (Ridge, 1972); in isolated perfused rat heart 2.7 (Hearse, Chain, 1972). Compared to the above values, the ratio of 0.69 obtained in *Anodonta* seems to be rather low. However, it is quite understandable if we consider the generally very slow tissue activity of bivalves. The direction of changes of this ratio is of much more importance than its absolute value. If the ATP/ADP ratio decreases, so does the phosphorylating capacity, and the relative accumulation of ADP stimulates glycolysis. The increase of this value induces the reversal of glycolysis, i.e., glyconeogenesis, if the mitochondria are respiring (Racker, 1965).

Accordingly, if we bring mammalian organs into hypoxia or anoxia, a decrease of the ATP/ADP ratio can be observed, as shown in the liver of newborn rats (Ballard, 1970), in isolated rat kidney (Hems, Gaja, 1972), in rat brain (Ridge, 1972), and in isolated, perfused rat heart (Hearse, Chain, 1972). In contrast, this ratio did not decrease in *Anodonta* during 6 days of anoxia, what is more, it increased with about 46% the average of 6 days being 1.01 and decreased below the normal only by the 7th day.

A similar metabolic significance is attributed also to the ATP/AMP ratio which in mammals is even higher than the ATP/ADP ratio. It changes in mammalian tissues under anoxic conditions in the same direction as the ATP/ADP ratio, and evokes the same metabolic effects (Ballard, 1970). In *Anodonta* this parameter also behaved during anoxia in a way different from the expected one (Table 1).

The "energy charge" parameter (Atkinson, Walton, 1967; Atkinson, 1968) indicates the quantitative energetic state of the tissues. Its value is about 0.8 in mammalian tissues, which always have a high ATP/ADP ratio. Anoxia induces a rapid decrease of the energy charge in the brain, liver and isolated kidney (Ridge, 1972; Ballard, 1970; Hems, Gaja, 1972). In *Anodonta cygnea* the value of "energy charge" was 0.43 during normal respiration and the same value could be calculated for *Mytilus galloprovincialis* (Zs.-Nagy, Ermini, 1972b). This is in accord with the relatively low energy demand of molluscan tissues and the low ATP/ADP ratio. Instead of a decrease of this parameter in *Anodonta* during anoxia it increased till the 3rd day and became lower than the normal only by the 7th day of anoxia.

The question arises which type of energy yielding mechanism is able to produce the extreme anoxic tolerance of *Anodonta*. If anaerobic glycolysis or glycogenolysis were responsible the following phenomena should be observed.

1. The carbohydrate consumption must then be increased to several times the normal (Lehninger, 1965). This was by far not the case. The carbohydrate consumption of other molluscs increased under anoxic conditions only moderately (Brand, 1946; Brand et al., 1950) or not at all (Chapheau, 1932; Meenakshi, 1956, 1958). In *Mytilus edulis* increased consumption has been demonstrated only in the adductors and hepatopancreas, which was, however, much lower than expected when calculated for the total animal (De Zwaan, Zandee, 1972b).

2. Lactate production should be increased. In contrast to this expectation in 20 species of *Gastropoda* Brand et al. (1950) could account for only 1–12% of the carbohydrates consumed in anoxia by the amount of lactate produced. Furthermore, Dugal and Fortier (1941) in *Ostrea*, as well as Wernstedt (1944) in *Dreissensia*, failed to observe anaerobic lactate production. A moderate formation of lactate was detected in *Venus* (Dugal, 1939) and in *Ostrea* (Humphrey, 1949). *Mytilus edulis* did not produce more lactate in anaerobiosis (De Zwaan, Zandee, 1972b).

Consequently, one cannot accept the assumption that anaerobic glycolysis alone would be able to maintain a sufficient energy supply during prolonged anoxia. It is possible that the pathway known in *Ascaris* (cf. Bueding, 1962) the end-product of which is succinate, plays some role also in *Molluscs*. Stokes and Awapara (1968) found equimolar amounts of succinate and alanine formed during anaerobic conditions in the mantle tissue of *Rangia cuneate* (Bivalvia). De Zwaan and Zandee (1972b) observed the increased production of succinate but not of alanine during anoxia of *Mytilus edulis*. The total amounts of succinate, alanine and D-lactate formed during anoxia account only for 50% of the carbohydrates consumed.

According to our assumption, the carbohydrates ingested during anoxia may be metabolized totally or partly via "anoxic endogeneous oxidation" (Zs.-Nagy, Ermini, 1972b). This mechanism can realize theoretically at least 57% of the energy content of carbohydrates, i.e., the energy demand of the tissues can be met in anoxia by only twice as much carbohydrate as required during normal respiration. Thus one can explain the increase of phosphorylating potential of the tissues in anoxia even without any significant increase of carbohydrate consumption. This mecha-

nism can obviously function only for a limited period of time. The limitation is certainly not due to the exhaustion of carbohydrate reserve, since a considerable part of them is available even at the end of anoxic tolerance. The limitation is probably related either to the exhaustion of the electron acceptor capacity or to the inhibition of reproduction of the electron acceptor substance. "Anoxic endogenous oxidation" most probably proceeds in the yellow pigment granules called cytosomes of molluscan tissues (Zs.-Nagy, 1971b, 1973a; Zs.-Nagy, Kerpel-Fronius, 1970; Zs.-Nagy, Borovyagin, 1972). This organelle can form the necessary compartments to accommodate the electron transport processes.

It should be noted that this mechanism is considerably more developed in *Anodonta* than in *Mytilus*. In the former the cytosomes are much larger and occur in all basic tissues (Zs.-Nagy, 1973a), whereas in the latter much smaller and fewer cytosomes appear (Zs.-Nagy, 1971a). This can be the reason why the former shows markedly better anoxic tolerance.

It has also been suggested that the anoxic tolerance of molluscs is based on the storage of oxygen by carotenoids of the cytosomal lipochrome pigment (Karnaukhov, 1971a, 1971b). It is shown elsewhere that this assumption in untenable on quantitative grounds (Zs.-Nagy, 1973b).

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A Theoretical Model for Calculation of the Rate Constant of Enzyme-Substrate Complex Formation

I. Calculation of Rate Constant in the Case of Motionless Enzyme Molecule without Nonspecific Intermolecular Forces

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The association rate constant of enzyme–substrate complex is discussed on the basis of a simple liquid model (Somogyi, 1971).

Only the special case is considered when the enzyme molecules are much larger than the substrates, and the nonspecific intermolecular forces are negligible. In this case the association rate constant of enzyme–substrate complexes can be determined by the aid of the model.

Introduction

Under certain conditions the rate of formation of enzyme–substrate (*ES*) complex can be determined experimentally (Chance, 1957; 1960; Ree et al., 1961; Turner et al., 1972). However in most of the cases there is no appropriate method to follow the formation of *ES* complex.

A theoretical model, which can be used to determine the rate of complex formation, may reveal the mechanism of enzyme action if we adjust the parameters in the model so that the computed rate constant should equal the measured one.

When the above constant cannot be determined experimentally the order of magnitude of the computed value may still give information about the kinetics of the enzyme.

A great number of models exist for the theoretical determination of the association rate constants of enzymes and substrates (North, 1964; Schurr, 1970a). The present paper deals with a model which although belongs to the group of so called "collision" models, but also differs from them as it works with some new parameters and handles others in a novel way.

The reaction to be dealt with is:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + Pr$$

where the k_1 and $k_{-1}\,k_2$ are the formation and decomposition rate constants of the enzyme–substrate complex, respectively.

This paper, the first of a series, treats only

$$E + S \longrightarrow ES \tag{1}$$

i.e., the first step of the above reaction.

Parameters influencing the rate constant

Rate constant k_1 which determines the rate of reactions like Eq. (1), can be expressed in terms of the following molecular parameters:

$$k_1 = \frac{qV}{\bar{t}} e^{-c_s V} \tag{2}$$

where c_s denotes free substrate concentration in moles/volume, V is the recognition volume, q and $\bar{t}$ are recognition probability and recognition time, respectively (Somogyi, Damjanovich, 1971). The recognition volume V, is a volume assigned to the binding sites of enzyme molecule as follows: within this volume the substrate can be bound by the enzyme molecule as a result of specific forces acting between the two molecules, but if the substrate is outside this volume the specific forces are ineffective. It should be noted that the substrate is in the recognition volume only if all parts of it are within this volume. The substrate molecule does translational and rotational diffusional motion in the recognition volume while the enzyme molecule is motionless. As the specific intermolecular forces between enzyme and substrate are effective over a distance of a few Å only, the size of the recognition volume is limited (Pollard, 1961). If the radius of a sphere comprising the substrate molecule is taken to be 5 – 10 Å, then the size of recognition volume is very likely the same as that of the substrate.

The recognition time ($\bar{t}$) is the mean time of occurrence of substrate in the recognition volume, when the substrate is not bound by the enzyme.

The q denotes the probability factor that the substrate becomes bound by the enzyme during the recognition time, i.e., before leaving the recognition volume.

It should be examined now, what is the connection between recognition probability and certain molecular parameters of an enzyme.

For the sake of simplicity we make some additional assumptions:

I. The substrate can be bound by the enzyme only if its kinetic energy is higher than ${}^{q}E$ threshold energy (this threshold energy may be equal to zero).

II. The substrate present in the recognition volume can be bound by the enzyme only if it turns into a suitable position by rotational diffusion.

III. If the above two conditions hold, the probability of formation of ES complex equals unity.

In the following we will consider these assumptions in the light of a simple liquid model (Somogyi, 1971):

There is a three-dimensional Cartesian coordinate system which builds up a lattice network. The molecules of liquids reside in the lattice points. The molecules do rotational and vibrational motions at each lattice point for τ average time.

There are six neighbouring lattice points for each molecule to be entered within a time negligibe as compared to τ.

If λ and D are the distance of two neighbouring lattice points and the diffusion constant of a molecule, respectively, then τ can be expressed as follows (Somogyi, 1971):

$$\tau = \frac{\lambda^2}{6D} \tag{3}$$

The size of λ, i.e., the distance between two nearest lattice points can be assumed to equal the distance between the molecules of quasi-crystalline water.

If we represent the position of molecules in space by coordinates of their mass-centers the above liquid model is able to describe the translational diffusional motion even if the diameter of sphere-like molecules in solution is $d > 2\lambda$.

According to this liquid model recognition volume is regarded as a specific order of some lattice points. (The magnitude of the distance between neighbouring lattice points within the recognition volume is also λ.) The substrate is in a suitable steric position for binding at every lattice point in the recognition volume when the line determined by the appropriate binding points of the enzyme and substrate crosses the center of substrate, i.e., the lattice point at which the substrate is actually found. In addition we assume that the substrate is also in a suitable position, when the binding point is within a circlet of its surface with its centre fitting the above defined optimal substrate point. The r_0 radius of this circlet is limited by the short range of action of specific intermolecular forces responsible for the formation of ES complex and is probably a few Å (Pollard, 1961). Then according to assumptions I to III and in respect of the whole surface of substrate we can express recognition probability leaving out of consideration the rotational diffusional motion of substrate molecules as follows:

$$q = \frac{r_0^2 \pi}{4\pi \rho_s^2} e^{-qE/kT}$$

where ρ_s denotes the radius of sphere-like substrate molecule, k is the Boltzmann constant and T is the absolute temperature. (The numerator and the denominator of the pre-exponential factor in the above expression are the measure of the successful events and of sample space, respectively.) When the rotational diffusion of substrate is also taken into account another question arises: how much of the substrate surface is covered by this special area while the substrate rotates during the recognition time. In case of rotational diffusional motion the mean square rotary angle of substrate molecule ($\bar{\alpha}^2$) during $\bar{t}$ recognition time (Setlow, Pollard, 1962) is

$$\bar{\alpha}^2 = 2\Theta_s \bar{t} = \frac{kT}{4\pi \eta \rho_s^3} \bar{t} \tag{4}$$

where Θ_s is the rotational diffusion constant of substrate and η is the viscosity.

It can be shown that a molecule (for instance the substrate) has to diffuse a distance 10 times its radius in order to present every possible aspect of its surface (Setlow, Pollard, 1962).

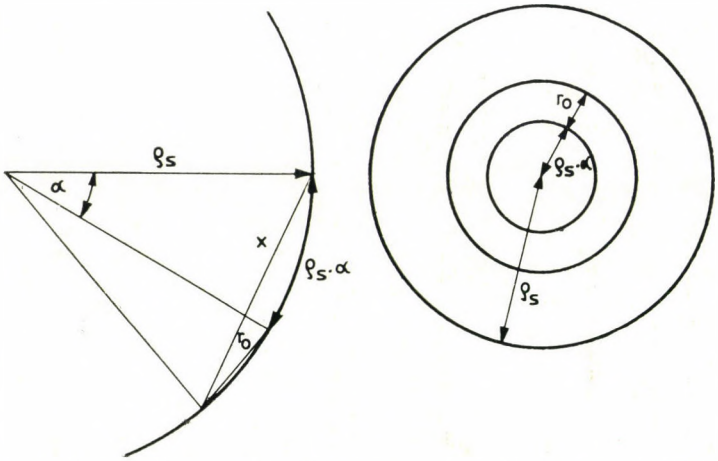

Fig. 1. The rotation of substrate molecule in the recognition volume. Any point of the area of the substrate of radius $\rho_s\alpha$ can be recognized if the center of this area is at the optimal position of the substrate. Because of the specific forces responsible for *ES* complex formation this radius is enlarged with r_0

Because there is little difference between the size of free volume of substrate and recognition volume, the mean square rotatory angle of substrate molecule is small during the recognition time. As demonstrated in Fig. 1, the differences between the appropriate arcs and chords are very likely negligible.

On the basis of the above assumptions one can write that area f on the substrate surface which is covered by the $r_0^2\pi$ territory during the recognition times is

$$f = \pi x^2$$

where

$$x = \rho_s\alpha + r_0$$

Now the recognition probability can be expressed if the rotational diffusion of substrate molecule is also taken into account:

$$q = \frac{\pi(\rho_s\alpha + r_0)^2}{4\pi\,\rho_s^2}\, e^{-qE/kT} \tag{5}$$

It is known that the D translational diffusion constant can be written according to the Einstein–Stokes equation as it follows:

$$D = \frac{kT}{6\pi\,\eta\rho} \tag{6}$$

where ρ is the radius of diffusing molecule and other symbols are the same as in Eq. (4).

Now substituting (4) and (6) into (5) we get:

$$q = \frac{1}{4\rho_s^2}\left(\sqrt{\frac{3}{2}\,D_s\bar{t}} + r_0\right)^2 e^{-qE/kT} \tag{7}$$

The recognition time can be determined as follows. First we introduce some new symbols:

M – is the number of lattice points in the recognition volume, i.e., the lattice points of recognition volume.

P_{ib} – is the probability that the substrate enters the recognition volume so that it gets into the i-th lattice point of recognition volume.

P_{i0} – is the probability that the substrate in the i-th lattice point of the recognition volume leaves it in such a way that it enters a lattice point outside the recognition volume.

P_{ij} – is the probability that the substrate, present in the i-th lattice point, when leaves it enters the j-th neighbouring lattice point. If there is free diffusion for the substrate according to the applied liquid model, $P_{ij} = 1/6$ for every j.

P_j – is the probability that a substrate molecule can be found in the j-th lattice point.

τ – is the average time while the substrate can be found in one lattice point.

r_k – is the number of lattice points of the recognition volume from which the substrate can leave this volume in the shortest way so that it makes not more than k jumps ($k = 1, 2, 3, \ldots$).

$r_1 = m$ – is the number of boundary lattice points, i.e., the number of lattice points of recognition volume which have at least one neighbouring lattice point outside the recognition volume.

S_i – is the number of neighbouring lattice points of the i-th boundary lattice point which are outside the recognition volume.

$'P_i$ – is the probability that the substrate leaves the recognition volume after it has taken exactly i jumps in the recognition volume.*

In connection with r_k one can write:

$$m = r_1 \leq r_2 \leq \ldots \leq r_k \leq r_{k+1} \leq \ldots \leq r_N = r_{N+1} = \ldots = M \tag{8}$$

if every boundary lattice point has neighbouring boundary lattice points. If only a single boundary lattice point exists, then $r_k = 0$ for any even k and according to this the expression (8) is valid for odd k's only. It is obvious that τ_i – the mean time of occurrence of substrate in the recognition volume during which the substrate makes i jumps – can be written as

$$\tau_i = i\tau \tag{9}$$

Consequently we can write for recognition time:

$$\bar{t} = \tau \frac{\sum_{i=1}^{\infty} {}'P_i \cdot i}{\sum_{i=1}^{\infty} {}'P_i} = \tau v \tag{10}$$

* We define a jump of the substrate to be within the recognition volume if it leaves a lattice point in the recognition volume.

where v is the average number of jumps made by the substrate during the recognition time in the recognition volume. Now we write probability $'P_i$ in a detailed form according to the previous definitions:

$$'P_1 = \sum_{i_1=1}^{M} P_{i_1 b}\, P_{i_1 0}$$

$$'P_2 = \sum_{i_1=1}^{M} P_{i_1 b} \left(\sum_{i_2=1}^{M} P_{i_1 i_2}\, P_{i_2 0} \right)$$

$$\vdots$$

$$'P_n = \sum_{i_1=1}^{M} P_{i_1 b} \left\{ \sum_{i_2=1}^{M} P_{i_1 i_2} \left[\sum_{i_3=1}^{M} P_{i_2 i_3} \left(\cdots \sum_{i_{n-1}=1}^{M} P_{i_{n-2} i_{n-1}} \left(\sum_{i_n=1}^{M} P_{i_{n-1} i_n} P_{i_n 0} \right) \cdots \right) \right] \right\} \tag{11}$$

In Eq. (11) there are defined but undetermined parameters such as P_{ib} and P_{i0}. It can be seen that for these we can write:

$$P_{ib} = \frac{\sum_{j=1}^{Si} P_j\, P_{ji}}{\sum_{i=1}^{m} \sum_{j=1}^{Si} P_j\, P_{ji}} \tag{12}$$

and

$$\text{for the boundary lattice points } P_{i0} = \sum_{j=1}^{Si} P_{ij} \tag{13a}$$

$$\text{for other lattice points } P_{i0} = 0 \tag{13b}$$

According to the above one can write $'P_n$ in another form:

$$'P_n = \sum_{i_1=1}^{m} P_{i_1 b} \left\{ \sum_{i_2=1}^{r_{n-1}} P_{i_1 i_2} \left[\sum_{i_3=1}^{r_{n-2}} P_{i_2 i_3} \left(\cdots \sum_{i_{n-1}=1}^{r_2} P_{i_{n-2} i_{n-1}} \left(\sum_{i_n=1}^{m} P_{i_{n-1} i_n} P_{i_n 0} \right) \cdots \right) \right] \right\} \tag{14}$$

Eqs (11) and (14) are the more applicable forms of $'P_n$ for the determination of its numerical value with a computer programme and with a table-calculator, respectively. By assuming that the substrate molecules in the solution obey Poisson distribution we can write for P_j (Maniloff, 1969):

$$P_j = \lambda c_s\, e^{-\lambda^3 c_s} \tag{15}$$

Now all the parameters are available that are necessary for the determination of the formation rate constant of an ES complex. With the aid of Eqs (2), (3), (4), (6), (7) and (10) after some algebraic rearrangement we get:

$$k_1 = 2V\, \Theta_s \left(\frac{1}{2} + \frac{r_0}{\lambda \sqrt{v}} \right)^2 e^{-qE/kT} \cdot e^{-V c_s} \tag{16}$$

where Θ_s is the rotational diffusion constant of substrate molecule according to Eq. (4). The value of v can be calculated on the basis of Eqs (10), (11), (12) and (13) in the easiest way by a computer. This computer calculation of v may yield an approximation only, but a fairly good one. Therefore the goodness of the approximation of k_1 value calculated by the aid of Eq. (16) is limited by the other estimations, such as the sizes of parameters λ, V and r_0.

Discussion

For the relationship between association rate constant, viscosity of solution and temperature Eq. (16) gives an explicit expression, as the dependence of rotational diffusion constant of the substrate upon viscosity and temperature is given in Equation (4). In relation to the other symbols in Eq. (14), we remark that the value of r_0 is roughly the same for all enzyme molecules, but quantities v, V and ${}^qE$ are characteristic of every enzyme and substrate system. The factor $\exp(-Vc_s)$ can be taken as 1, since the order of magnitude of recognition volume is the same as the free volume of substrate molecule in the solution (Somogyi, Damjanovich, 1971).

Then if we take ${}^qE$ nearly zero or assigning a value to it, k_1 can be calculated according to the Eq. (16) as follows: If we have some information about the action range of specific forces, the size of substrate molecule and the geometry of binding site of the enzyme, the recognition volume can be estimated. Representing the position of substrate in space by the coordinates of its mass center the value of v can be calculated.

Knowing the values of Θ_s, r_0 and λ we can determine the value of k_1 on the basis of Eq. (16).

As regards the limits of the presented model we note the following: The approximation of the calculated value of k_1 is limited by the inaccuracy of the values of V, λ and r_0. But if the exact value of k_1 can be determined experimentally, one can take a correction for the above parameters bringing the calculated value of k_1 closer to the measured one.

In this way we can get some new information about the binding center of the enzyme molecule.

Further the model only considers the case when the formation of a single bond between the enzyme and substrate molecules is responsible for the formation of an enzyme-substrate complex. However, from our consideration we can say that the model is correct also if the bonds (three bonds are assumed between the enzyme and substrate molecules because of the stereo-specificity of enzymes) are formed at the same time or in an ordered way. In the latter case the model is valid if the bond formed does not break up before the formation of all bonds. In any other case, the model is able to describe only the formation of the first complex defined as the formation of the first bond between the enzyme and substrate molecules.

The assumption that the enzyme molecule does not move during the recognition process is also an oversimplification.

However, the model is valid for systems where the enzyme molecule is much larger than the substrate. In such cases the diffusional motion of enzyme molecule is indeed negligible as compared to the diffusion of substrate.

Finally, it should be emphasized that this model is a very simple one, since we neglect the diffusional motion of enzyme and the nonspecific intermolecular forces between the enzyme and substrate molecules. These problems will be discussed in a forthcoming paper.

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The Effect of the Spectral Composition of Light on the Metabolism

Utilization of Light and the Chlorophylls of *Sinapis alba*

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The synthesis of nitrogen compounds is specifically stimulated by blue light, that of carbohydrates is independent of the spectral composition of light. The amount of chlorophyll-a does not depend on the spectral composition of light, however, in ageing plants the chlorophyll complexes belonging to the two photosynthetic systems decompose in different amounts. In determining the fluorescence spectra *in vivo* the importance of the correction for reabsorption of fluorescence is emphasized.

Introduction

It is known that the intensity of photosynthesis is influenced by the "colour", i.e., by the spectral composition of light and, due to the intensity of photosynthesis, also the products of photosynthesis are affected. Therefore, in studying the effect of the spectral composition of light on the products of photosynthesis the same intensity of photosynthesis should be ensured in experiments with light of different spectral composition. This condition can be realized by applying different intensities of light during the treatments with lights of different composition. In addition, this condition leads to different intensity ratios of lights of different colour in case of various plants.

This point of view is very often overlooked, therefore, in this paper the effect of red and blue light on the accumulation of carbohydrates and nitrogen compounds and also on the pigment system of *Sinapis alba* has been studied under the same intensity conditions.

Experimental

The plants were cultured under controlled conditions for three weeks. Fluorescent tubes were used as light sources, the intensities of blue and red light were 25 000 and 14 000 erg $cm^{-2}sec^{-1}$, respectively.

The blue light consisted of 13 per cent below 436 nm, 39 per cent from 436 to 495 nm, 32 per cent from 495–655 nm and the rest above 566 nm; the red light

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was composed of 89 per cent above 627 nm, 10 per cent from 588–627 and the rest below 588 nm. In this case the accumulation of dry matter was found to be practically the same for the two light conditions. The duration of illumination was 16 hours/day. During illumination and in darkness the temperature was 25–30 and 20–25°C, respectively.

The absorption spectra of leaves *in vivo* were measured at room temperature with a Unicam SP 800 recording spectrophotometer supplied with a sample holder position for highly scattering samples. The residual scattering was corrected by subtracting the apparent optical density (due to the scattering) at 750 nm.

The fluorescence spectra of leaves *in vivo* were measured at room temperature with a photoelectric device built in this laboratory. The exciting light was produced by a high pressure mercury lamp and the wavelength of 436 nm was selected with a metal interference filter. The fluorescence was observed through a Zeiss monochromator SPM–2 with a photomultiplier EMI 9558 A. The intensity of illumination measured calorimetrically was of the order of 10^1f photon/cm^2 sec.

Results and discussion

a) *Metabolism and utilization of light.* The results of the analysis of the leaves of plant are shown in Table 1. This table shows that the synthesis of nitrogen

Table 1

Light	Dry weight		Carbohydrates				Nitrogen compounds			
			soluble		starch		soluble		protein	
	mg	%	γ/mg	%	γ/mg	%	γ/mg	%	γ/mg	%
Red	15.2	100	50.0	100	40.4	100	7.6	100	36.8	100
Blue	16.2	106	50.8	102	40.8	101	8.6	113	45.2	123

compounds, first of all that of proteins, is specifically stimulated by blue light (th stimulation is specific in a sense that the differences of syntheses under blue and red light cannot be attributed to differences in the accumulation of dry matter which actually have been eliminated by using different light intensities). This is in accordance with earlier observations of a direct stimulation of the synthesis of proteins in blue light (Klesnin et al., 1955, 1960). If the soluble fraction of nitrogen would exhibit higher differences, a general stimulation of the metabolism of nitrogen could be assumed. The response of plants to the spectral composition of light is different in a sense that the synthesis of nitrogen compound is not always influenced by blue light, e.g., in *Vicia faba*. In this case, however, an accumulation of starch could be detected.

The light treatments were applied at different intensities of light (adjusted to the same amount of production of dry matter).

For a comparison of the effect of light the absorbed energy of light should be compared. Therefore, the light absorption of leaves *in vivo* and the leaf area should be considered. The incident and absorbed energies are summarized in Table 2.

Table 2

Light	Incident energy		Absorption of leaves 400—700 nm %	Area of leaves cm^2	Absorbed energy	
	10^3 erg	10^{15} photon			10^3 erg	10^{15} photon
	cm^2sec^{-1}				cm^2sec^{-1}	
Red	14	4.58	27	14.3	54	17.7
Blue	26	6.16	22	13.0	72	17.6

The absorbed energy is higher in blue than in red but there is no difference in the number of photons. Using the data of Table 2 the relative utilization of the energy of light in the production of organic matter can be calculated as given in Table 2. The utilization of blue light relative to that of red light is less if the comparison is made by considering the absorbed energy. However, for a comparison considering the absorbed number of photons, there is practically no difference in blue and red light. This observation seems to corroborate the assumption that also the second excited singlet state is participating in photosynthesis (an "underheard-manner" behaviour according to Rabinowitch (Rabinowitch, 1964)), either directly or after radiationless transition to the first singlet state, since the light absorbed by carotenoids in this spectrum region cannot be responsible for the 95 per cent utilization (Table 3). If the utilization of energy (and not the number of quanta) is considered, the utilization of blue light is poorer than that of red light.

Table 3

Light	Relative utilization of light			
	to incident		to absorbed	
	energy	number of photons	energy	number of photons
Red	1.00	1.00	1.00	1.00
Blue	0.52	0.68	0.69	0.95

According to Kowallik (Kowallik, 1966) the action spectrum of the synthesis of proteins runs parallel to the absorption spectrum of carotenoids in the blue region, therefore, in our work the stimulation of the synthesis of proteins by blue light may be due to the role of carotenoids in the synthesis.

The poor utilization of light absorbed by carotenoids compared to that absorbed by chlorophylls was demonstrated with measurements of the quantum yield of photosynthesis (Emerson, Lewis, 1943).

b) *The chlorophylls and the spectral composition of light.* Under the same conditions as before the effect of the spectral composition of light on the chlorophylls was studied by using the absorption and fluorescence spectra of leaves *in vivo*.

The red bands of the absorption spectra of plants grown under "blue" and "red" light are shown in Figs 1 and 2 at the ages of 1, 3 and 5 weeks. $k(\lambda)_{max}$ – the optical density at the maximum, $\Delta\lambda$ – the half band widths of red band are summarized in Table 4. The optical density in the first three weeks is practically inde-

Table 4

Light	1st week		3rd week		5th week	
	$k(\lambda)_{max}$	$\Delta\lambda nm$	$k(\lambda)_{max}$	$\Delta\lambda nm$	$k(\lambda)_{max}$	$\Delta\lambda nm$
Blue	1.99	30.0	1.28	28.0	0.66	28.0
Red	1.93	30.0	1.27	28.0	0.14	28.0

pendent of the quality of light but in the last period of time it strongly decreases in red light and less in blue one. The half band width decreases with time; this decrease should be attributed to the different rate of decomposition of the chlorophyll-a components belonging to the two pigment systems during senescence.

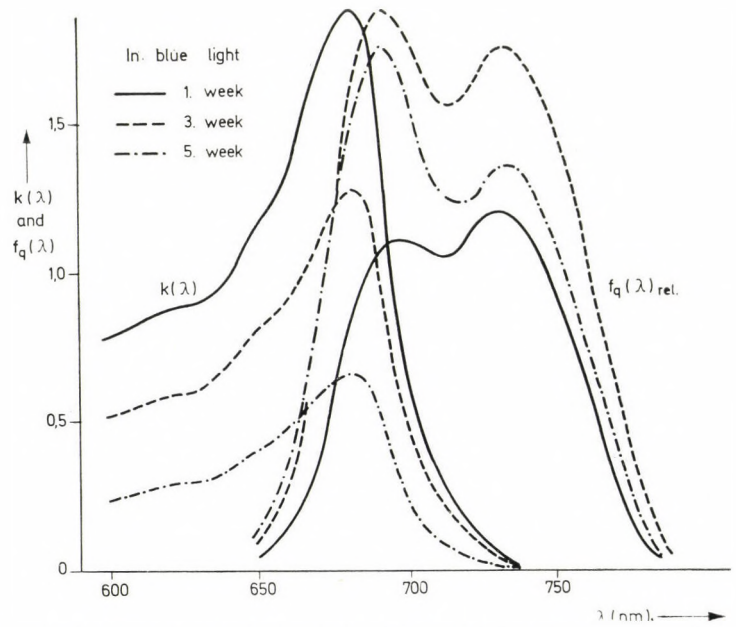

Fig. 1. Absorption and fluorescence spectra of leaves of *Sinapis alba* grown in blue light as a function of age

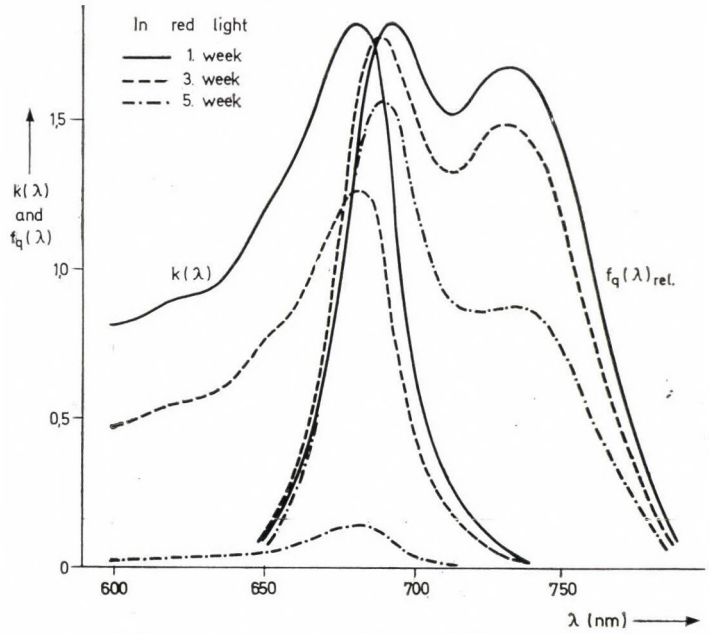

Fig. 2. Absorption and fluorescence spectra of leaves of *Sinapis alba* grown in red light as a function of age

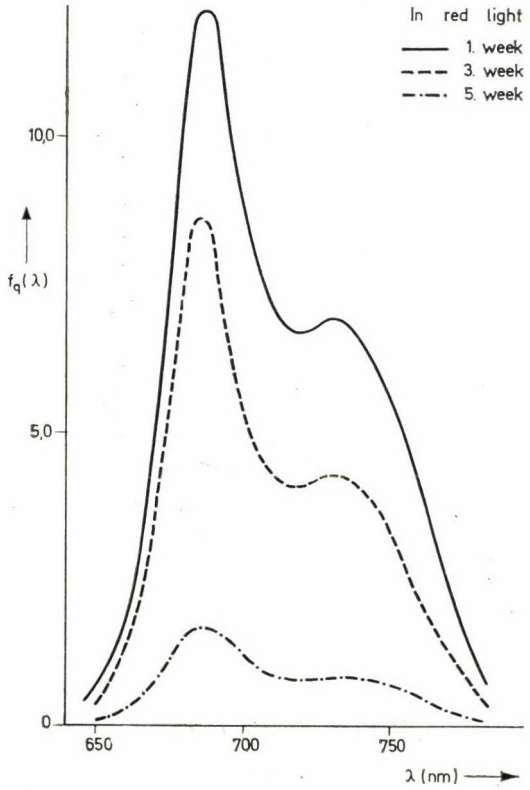

Fig. 3. Fluorescence spectra of leaves of *Sinapis alba* grown in blue light, corrected for reabsorption of fluorescence, as a function of age

The relative fluorescence spectra $f_q(\lambda)_{rel}$ shown in Figs 1 and 2 have two bands approximately of the same height at 690 and 740 nm. Since the long wave tail of the absorption band and the short wave band of the fluorescence strongly overlap the effect of the reabsorption of fluorescence should be considerably high. This is shown in Figs 3 and 4 for plants grown in red and blue light, respectively. In these figures the corrected spectra exhibit a high short wave band and a low long wave band in the fluorescence. On comparing the uncorrected fluorescence spectra with the corrected ones we observe a shift towards the longer waves caused by reabsorption (Rabinowitch, 1951). It is worth to mention that the intensity of fluorescence for plants grown under blue light increases in the first three weeks while that under red light decreases. This may be due to the differences in the ratio of the amounts of the two chlorophyll-a components during the cultivation period, the fluorescent component (Chl – 670) being decomposed slower (Raafat et al., 1969) than the nonfluorescent one.

Figs 5 and 6 show the fluorescence spectra normalized to the unit area. These spectra offer a possibility of the comparison of the fluorescence spectra in plants of different age, since here only the spectral distributions are kept and the intensities are omitted. On ageing the short wave band increases, the long wave

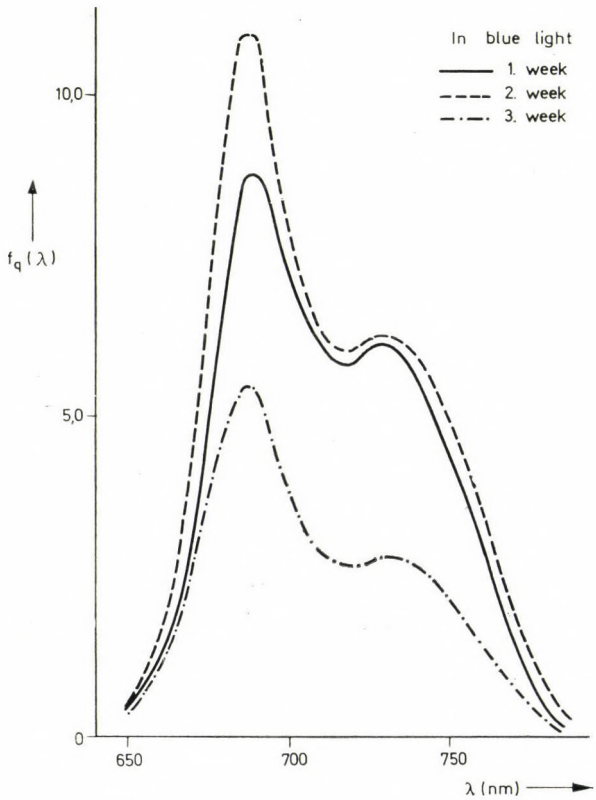

Fig. 4. Fluorescence spectra of leaves of *Sinapis alba* grown in red light, corrected for reabsorption of fluorescence, as a function of age

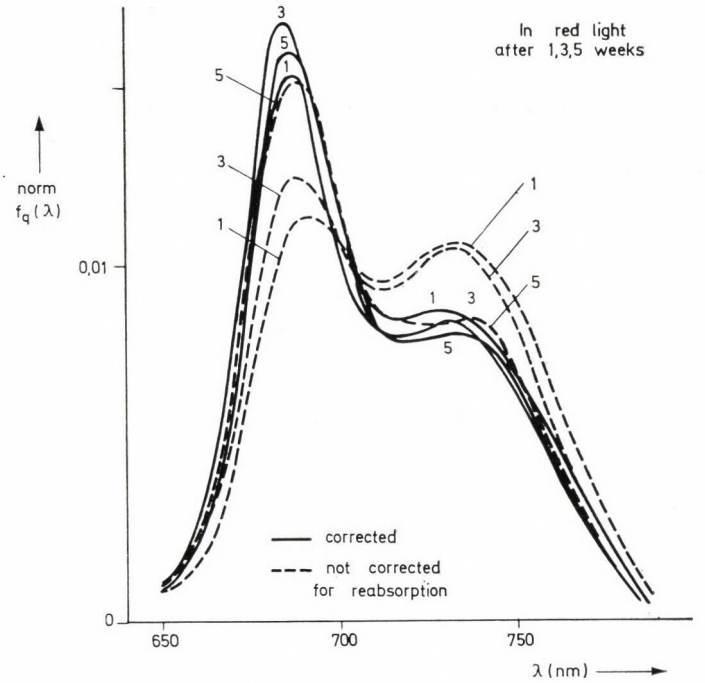

Fig. 5. Normalized fluorescence spectra of leaves of *Sinapis alba* grown in red light, as a function of age

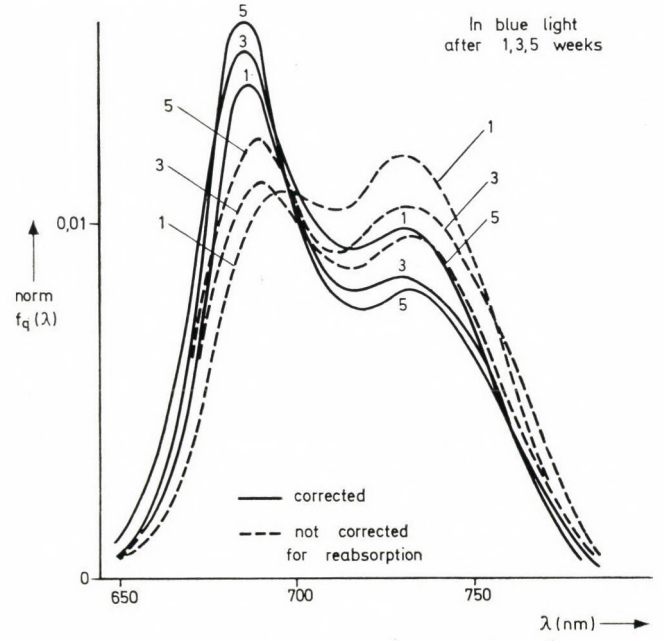

Fig. 6. Normalized fluorescence spectra of leaves of *Sinapis alba* grown in blue light as a function of age

band decreases both for uncorrected and corrected spectra, but in the latter case the deviations are rather tendencies than pure facts (the differences are less than 10 per cent). Since the eventual sieve-effect and the dependence of the scattering of light on the wave length in the absorption spectra were not considered, these small deviations cannot be safely attributed to a true phenomenon (except the decrease of the long wave band for plants grown under blue light).

As estimate of the relative concentration of the fluorescent Chl−670 component was attempted by using the maximum optical density $k(\lambda)_{max}$ (as a measure of the total concentration of both Chl-a components) and the intensity of fluorescence $I(\lambda)$ (as a measure of the concentration of the fluorescent Chl−670 component) c_{fluor} according to (Raafat et al., 1969):

$$c_{fluor} = \text{const}\, I(\lambda) \cdot k(\lambda)_{max}.$$

The values of $I(\lambda)$ calculated from the relative areas of the corrected spectra shown in Figs 3 and 4 are summarized in Table 5. Using these values and the values of $k(\lambda)_{max}$ from Table 4 the relative concentrations of both components for all cases are obtained (Table 6).

Table 5

Light	1st week	3rd week	5th week
	$I(\lambda)$	$I(\lambda)$	$I(\lambda)$
Blue	0.79	0.92	0.44
Red	1.00	0.65	0.14

Table 6

Light	1st week		3rd week		5th week	
	c_{fluor}	$c_{non\text{-}fluor}$	c_{fluor}	$c_{non\text{-}fluor}$	c_{fluor}	$c_{non\text{-}fluor}$
Blue	0.80	0.80	0.59	0.43	0.14	0.38
Red	1.00	1.00	0.47	0.86	0.10	0.04

It can be concluded that the decomposition of the fluorescent component is approximately uniform in blue light, while the non-fluorescent component practically does not decompose in the last period (3−5 weeks). In red light both the fluorescent and non-fluorescent components show a decomposition with the age, with an especially rapid decomposition of the non-fluorescent component seen in the last period of time.

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Survey of Muscle Biophysics*

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I. The striated muscle is a *macroscopic machine* endowed with all the characteristics corresponding to the physical definition of a machine. Namely muscle a) performs motion or changes the direction of motion; b) exerts force, thus performs work during motion; c) uses energy or transforms it while working. The machine-like activity of muscle constitutes its social importance, on the one hand, and receives appreciation from the point of view of the state of health of the whole population, on the other. This great social importance of the striated muscle has not come across appropriately in the basic research either in the past, or in the last few decades, and thus the international production in the field of muscle mechanics seems to be out of proportion.

II. The muscle-machine is naturally a research field of biophysics, but is being investigated to a large extent also by biochemists; accordingly we are witnesses to an abundance of biochemical literature e.g. on muscle proteins. In 1940 I asked Szent-Györgyi whether he really considered myosin and actin as two separate, independent contractile proteins. The answer was positive. Thirty years later (Ernst, 1970) I mentioned about 10 "contractile proteins" in a paper written at the request of the Editor and I remarked that many more could be described in the future. Namely, when the molecular weights of myosin and amino acids are – in the order of magnitude – 10^6 and 10^2, respectively, then myosin can be looked upon as consisting of 10^4 amino acids. Thus – I continued – different parts can be cut off by different methods from myosin, their number being

$$N = n - (m - 1),$$

where $n = 10^4$, and $m =$ the number of amino acids in the pieces (of equal length and sequence). Accordingly $N = 9901$ and 9001 new molecules could be prepared with the mol. weights of 10^4 and 10^5, respectively. Today, 3 years later, I can enumerate many new "proteins" due especially to the IVth International Biophysical Congress (Moscow, 1972) and the 17th Biophysical Meeting (Colombus, Ohio, 1973). (Drabikowski, 1972; Watanabe, Staprans, Russell, 1972; Cooke, 1972; Kuehl, Conti, Adelstein, 1972; Eaton, Pepe, 1972; Sreter, Holzer, Gergely,

* Lecture delivered at the invitation of the Hungarian Biophysical Society on May 31, 1973.

Holzer, 1972; Madeira, Madeira, 1973; Meissner, Conner, Fleischer, 1973; Weisel, 1973; Brekke, Greaser, 1973; Szent-Györgyi, 1973; Gergely, 1973; Zobel, 1973; Dreizen, 1973; Murphy, 1973.)

III. 1. Biochemical aspects played an important role in muscle research also in the twenties totally neglecting structure (Ernst; 1928); today structure is dominant in myology neglecting many other important facts. The initial picture of *longitudinal filaments* of the fibril has been enlarged to pictures of 1-, 2-, 3-filamentous descriptions. At any rate our data (Ernst, Benedetzky, 1962) describing *longitudinal filaments continuously passing along* the length of the fibril (Fig. 1) were seemingly accepted by Hoyle (1973). According to our description these longitudinal filaments pass the *Z*-lines, better to say the *Z*-discs, described repeatedly by some authors without mentioning the first ones (Ernst, Guba, Garamvölgyi, 1958).

In good agreement with this picture of continuous longitudinal filaments is the picture (Garamvölgyi, 1966) demonstrating that the material of the thick filaments migrates towards the *Z*-discs along preformed filaments (Fig. 2). This picture fits in well with the new data (Squire, 1971) according to which the myosin molecules are packed into a surface layer the thickness of about 35 Å around a core filament.

These data are in contrast with Huxley's conception (Hanson, Huxley, 1955) in connection with which we (Ernst, Benedetzky, 1962) raised the question (p. 211): ". . .how could the *A*-filament – hovering in the A-space of the sarcomere – be affected by a stress acting upon the fibril?" After a few years we got the answer in the literature describing the *C*-filaments as connecting the *A*-filaments with the *Z*-discs. This agrees again with the conception of the continuous longitudinal filaments, according to the experimental results (Garamvölgyi, Belágyi, 1968) showing that a fibre from the wing muscle of the honey bee could be stretched even to 300 per cent. (On the other hand, the lecture of Garamvölgyi et al. claiming as a general opinion that the muscle material does not participate in the elasticity of the resting muscle seems to me quite ununderstandable. The more so, because at the same session Chaplain spoke about the extensibility of the filaments at rest and in rigor.)

The *transversal structure of the fibril should* also be mentioned (Ernst et al., 1969; Trombitás, 1971). Fig. 3 shows that the fractures of the fibril were mainly transverse with straight border-lines at right angles to the longitudinal axis.

III. 2. *In connection with the mechanism of contraction* as described by the *sliding-hypothesis* the question arises: how could the thick filaments of a sarcomere pull together the thin filaments and with them the two *Z*-discs bordering the sarcomere? Namely, among the three sarcomers in Fig. 4 the one in the middle (2) should pull the *Z*-discs Z_1 and Z_2 nearer to the middle of the sarcomere, but the two other sarcomeres (1 and 3) would pull the same *Z*-discs apart from sarcomere 2 simultaneously. Except for the case if the sarcomeres are supposed to perform their contractions in succession, but, in this case, the shortening of a single sarcomere of about one μ could not produce the macroscopical shortening of the muscle.

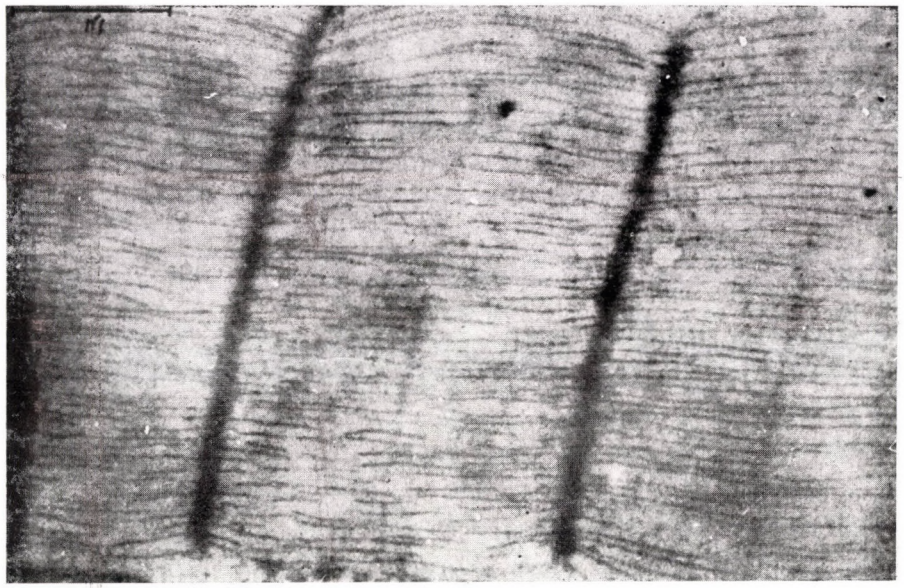

Fig. 1. Continuous filaments through the *Z*-discs (Ernst, Benedetzky, 1962)

Fig. 2. The material of the *A*-filaments migrated towards the *Z*-discs (Garamvölgyi, 1966)

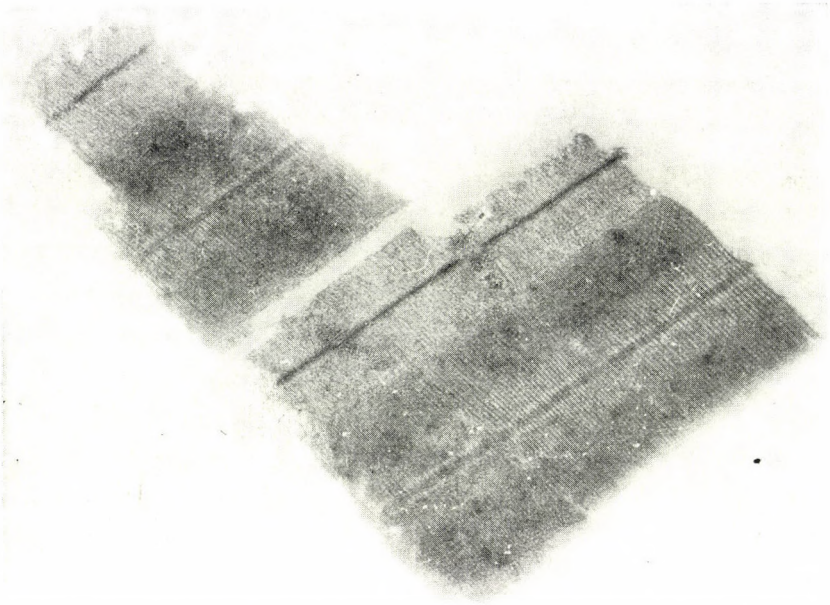

Fig. 3. Transversal fracture lines of the fibrils (Trombitás, 1971)

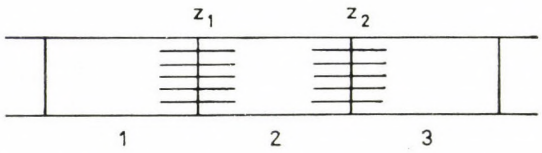

Fig. 4. Model of three sarcomeres (see text)

The birth of the sliding hypothesis was helped by a paper (Astbury, 1947) describing that the X-ray picture of a muscle, resting or contracting, did not show any change in molecular structure; this offered the basis of the hypothesis that the filaments only interdigitate during shortening and do not shorten. But it was and still is a mistake in principle that the *negative* result of an experiment would make one forget *positive* experimental facts having shown that the anisotropic bands (= *A*-filaments) do shorten during contraction. Anybody who has ever seen the spontaneous contractions of a fibre of the leg muscle of Hydrophilus (Fig. 5, e.g. Ernst, 1968) will remain immune to opinions contradicting experimental facts. Figs 6 and 7 are taken from Schmidt's papers (1937a, 1937).

The shortening of the *A*-band during contraction was repeatedly mentioned at both Congresses quoted above. Frank and co-workers (Samosudova et al., 1972) reported shortening of the thick filaments by 25 per cent; Herman and Dreizen (1972) also spoke about shortening of the *A*-band. These data were delivered at Moscow; the following ones in the USA: Dewey (1973) reported on shortening of the thick filaments by even more than 40 per cent, Dreizen (1973) by 10–15

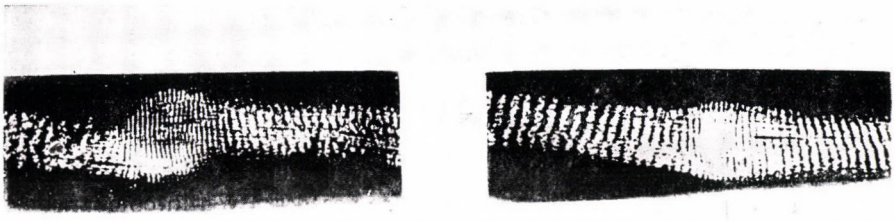

Fig. 5. Spontaneous contractions of a fibre from the leg muscle of Hydrophylus (Ernst, 1968) × Nicol, × 300

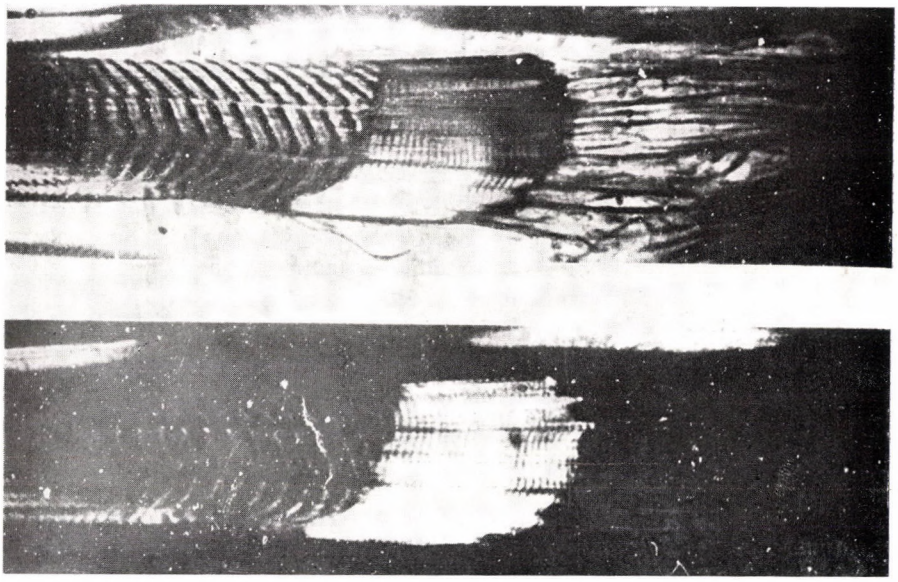

Fig. 6. Muscle fibre contraction of Cyclophorus lumpus (Schmidt, 1937a); a) × Nicol, b) compensated by 1/6 mica; × 650

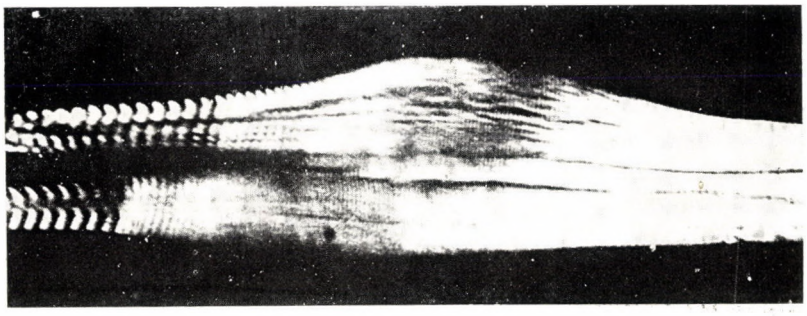

Fig. 7. Contraction of a muscle fibre from Dytiscus (Schmidt, 1937); × Nicol, × 150

per cent. If, however, it turns out that *some parts of the muscle do shorten during contraction*, then the consequence of the negative result of the investigation by X-ray diffraction loses its credit, and *the principal basis or motivation of the sliding hypothesis can be considered as having ceased to exist.*

Therefore it is of great interest to pay attention to Szent-Györgyi's activity which has produced – as generally known – one of the most basic conceptions in the field of muscle research in the last three decades. Even in comparison to his achievements his hypothesis (Szent-Györgyi, 1960) means a very important development and is in accordance with his general conception of electronbiology. According to this hypothesis an electron process between ATP and myosin generates (p. 119): ". . .an electric potential, a current. There is no difficulty in making theories of how such currents could produce contraction" and he continues (p. 120): ". . .this speculation allows me to make for the first time, after twenty years of muscle research, an intelligible picture of how ATP could drive muscle".

Szent-Györgyi's conception of the electric nature of muscle contraction interested me especially, because I simultaneously reported at the Muscle symposium at Pittsburgh (Ernst, 1960) and also at the Meeting of the Hungarian Biophysical Society that minute protein threads made of hen's egg white, fibrin, casein and gelatine contract in a medium containing KCl, $MgCl_2$ and ATP, and the ions and ATP play their role in this process just by means of their electric property (Ernst, Metzger, 1962).* It should be mentioned that the electric view of contraction was lent new support to at the Congress in Moscow (Fukada, 1972; Laki, Aliving, 1972; Teplova, Karnaukov, 1972; Troshina, 1972).

IV. 1. After chemistry, structure and mechanism, the *energetics of muscle follows;* in this respect metabolism or heat production comes into prominence. The thesis of Hill (1926) should follow first (p. 44): ". . .no means of separating the internal metabolic changes and the external mechanical changes has ever been found", and (p. 45): "Even the highest sensitivity of the methods available only showed that the line relating heat to tension passes through the origins". In contrast to that I repeatedly emphasized (Ernst, 1928, 1929) that the specific function and the metabolism of an organ must not be mixed up, thus the mechanical and metabolic processes of muscle should be analysed separately.

We succeeded in demonstrating (Ernst, Fricker, 1931) that muscle *stretched passively* produced about 1/3 of the maximum of lactic acid produced by a maximally tetanized muscle. The next year appeared the paper (Feng, 1932; Hill's laboratory) reporting that a muscle stretched passively, produced more heat; this so-called "Feng-effect" seems to Clinch (1968) to corroborate our result mentioned above. Thus it turned out that *metabolism or heat production could be analysed separately from the specific contractile function of muscle.* This conception was corroborated definitively by Hill himself (1957) stating that a muscle perfused with a hypertonic solution and stimulated electrically ceased to contract but produced heat.**

* Contrasting with these facts the thesis says: ". . .ATP does not produce any change in the shape of any other known protein" (sc. outside myosin. Weber, H. H., 1957).

** In such a state action current can be registered (Demoor, Philippson, 1908; Ernst, 1926).

IV. 2. The *heat production of the contracting muscle* is under lively discussion even today. Mostly because the terms "isotonic" and "isometric" do not – as already expounded by me earlier (Ernst, 1963) – properly differentiate between shortening (without force) and force (without shortening) (Weber, 1846). To this unelucidated situation can it be ascribed that the so-called heat of shortening is being widely debated, and the thesis according to which "isotonic" contraction is accompanied by greater heat production than "isometric": $h_{it} > h_{im}$, plays a role in the literature.

Neglecting methodologic details, the fact should be emphasized that Tigyi (1959) has demonstrated the contrary by the calorimetric method: $h_{it} < h_{im}$. And facts are stronger than formulations; thus in its text a paper claims (Auber, 1970) that isotonic heat is greater than isometric, but its table contains the measured result: "$h_{it} - h_{im} = -16$ units." That is similar to Tigyi's result, with which also newer data (Gibbs, Gibson, 1970; Matsumoto, McPhedron, 1972) are in agreement.

IV. 3. In connection with energetics, the debate concerning the *efficiency* of muscle work should also be mentioned; the basis of this debate is the circumstance that this physical notion is used but not understood by most biologists. Thus the relevant numerical values cannot be generally considered as acceptable.

V. Knowledge *of the real resting state of some parts in the muscle substance* is necessary to be able to estimate the importance of changes in the state accompanied by activity.

V. 1. *The state of water* was very often dealt with in the literature, and I joined (Ernst, 1926; 1926; 1925; 1928) those authors who – for want of a better expression – considered muscle water bound. In contrast to that muscle was regarded as an "osmotic sac", and in addition it was emphasized that muscle did not contain bound water (Hill, 1930). The problem seems to be definitively solved (Ernst et al., 1950) by the demonstration that the relative lowering of the vapour pressure of muscle during drying in a gaseous atmosphere changes similarly to a swollen system, i.e., it can be much greater than it would be if the muscle really represented an osmotic sac, i.e., if muscle water played but the role of a solvent. Today the literature seems to have generally accepted the view of bound water.

V. 2. *Bound potassium* was often treated of by me (e.g. Ernst, 1963) in accordance with the earlier literature in a positive sense (Ernst, Scheffer, 1927), and similarly resolutely negated by Hill (1930). The problem is being debated even today and will perhaps turn out to the advantage of bound potassium.

VI. While disregarding many other important phenomena of activity, *hypertrophy* may be mentioned as a property of the muscle machine, which surpasses all man-made machines by far. By analysing this side of the muscle machine we are led to the vast field of control processes in general and the recognition that muscle is only one part of a more complex biomechanism, e.g. the quick lever consisting of forearm, elbow-joint and biceps and its innervation.

A description of the other motional function of walking may now be attempted *from the point of view of biocybernetics* in a much simplified way. Accordingly the *matrix of transformation* should follow to describe this function as the alter-

nating activity of the two legs (Fig. 8, r_s = stretching of the right leg, r_b = bending of the right leg; l_s and l_b mean the same for the left leg). The cyclic sequence of the movements of the two legs is shown by the main diagonal (containing the figures 1) in this simplified example. Further, the connection of these activities with the nervous system may be symbolized in a simplified manner by the trajectory of Fig. 9 (sp_i and b_i = relevant parts of the spinal cord and the brain).

↓	r_s	l_b	l_s	r_b
l_b	1	0	0	0
l_s	0	1	0	0
r_b	0	0	1	0
r_s	0	0	0	1

Fig. 8. Matrix of transformation demonstrating the cyclic activity of the two legs

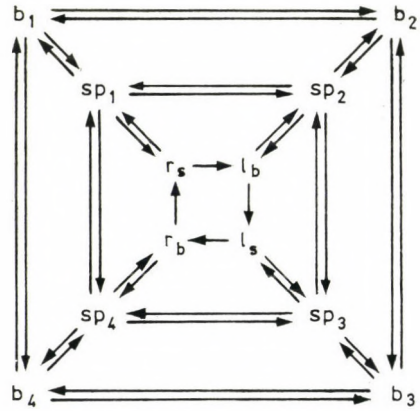

Fig. 9. Trajectory for further analysis of Fig. 8

This last exposition – primitive as it is – wishes to indicate that in connection with muscle research complex biomechanisms are to be analysed on the one hand, and this can be implemented only by means of interdisciplinary investigations, on the other.

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Abstracts

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1. Subject: *Molecular Mechanisms and Energetics of Muscle Contraction*

The Role of Water Transport in the Veratrine Induced Depolarization

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It has been observed in experiments on the depolarizing effect of veratrine that the muscle will swell after treatment with 0.1 mM of veratrine. The increase in wet weight of the sartorius muscle averaged 13 to 18 per cent during a 60 min veratrine treatment. This observation is in good agreement with earlier findings according to which the ^{24}Na influx will increase upon the effect of veratrine (Varga et al.: The effect of veratrine on the ion transport of frog skeletal muscle. Acta Physiol. Hung. In press). On the other hand, the present investigations indicate that the specific gravity of the muscle remains unchanged when the Na-uptake and wet weight of muscle increase. All these observations can be interpreted in such a way that an isotonic solution is taken up by the muscle upon the effect of veratrine.

Veratrine fails to depolarize in hypertonic solution of various compositions (normal Ringer + 150 mM of NaCl; normal Ringer + 300 mM of sucrose or glucose). On the basis of the data reported the assumption can be made that also water transport may play a part in the mechanism of veratrine induced depolarization.

A New Method for Continuous Extracellular Recording of Potential Changes in Skeletal Muscle

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To follow continuously the membrane potential changes of muscles treated with different drugs for several hours even during mechanical activity, is a task extraordinarily difficult to solve by intracellular measurements.

The method here reported provides a good possibility to solve this problem by applying an extracellular procedure. The method is based on the separation of the two parts of muscle by means of a rubber diaphragm. The diaphragm electrically insulates the surfaces of the muscle membranes separated by it and, in this way, it becomes possible to record the difference in the average membrane potentials of the two separate muscle parts from the incubation solutions. If the one part of muscle is immersed in normal Ringer solution the measured values practically show the potential changes of the other part.

The records obtained by this method mainly enable qualitative evaluation but, by using an appropriate calibration curve, the method might also be suitable for quantitative measurements.

The Effect of Ouabain on the Distribution of Sodium Ions in Striated Muscle

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It was found in the authors' earlier investigations that about 40 per cent of the total intracellular sodium was labelled with a higher rate than the remaining part in freshly dissected frog sartorius muscles stored in ^{24}Na Ringer solution (Szabó et al., Congress of the Hung. Physiol. Soc., 1972).

In the present experiments the influx and efflux kinetics of ^{24}Na ions were studied in normal and ouabain treated muscles. The net sodium uptake was increased by 10^{-4} M ouabain in muscles kept in normal Ringer solution. The fast equilibrating Na compartment was increased while the slowly equilibrating Na compartment was decreased by the drug. Unidirectional Na-influx increased non-linearly with increasing external sodium concentration between 0.15 and 111.5 mM Na in K-free isotonic solutions balanced with choline. The unidirectional sodium influx was unaffected by 5×10^{-5} M ouabain under K-free conditions.

The wash-out kinetics were studied using ouabain free media in muscles labelled with ^{24}Na in the presence or absence of the glycoside. The results indicate that the intracellular sodium compartmentalization can be altered by inhibition of the metabolically driven pumped fluxes. It is obvious that the inward leakage flux of sodium ions remains unbalanced in ouabain poisoned muscles; however, a direct effect of the drug on intracellular sodium binding cannot be excluded.

The Role of Calcium Ions in the Function of Electromechanical Coupling of Muscles

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In frog muscles equilibrated in normal Ringer solution the depolarization induced by high K-solutions is followed by contractures. If the calcium concentration of the Ringer and high K-solutions was reduced from 1.8 mM to 0.1 mM the amplitude and duration of potassium contractures reversibly decreased, depending on the duration of preincubation in high or low calcium-Ringer solution. 1 mM La^{+++} reduced the amplitude and increased the time necessary for the development of K-contractures. This effect of La^{+++} was studied in experiments carried out with fibre bundles of m. semitendinosus incubated in ^{45}Ca-Ringer solution. From the kinetics of the ^{45}Ca uptake and wash out in the presence and absence of 1 mM La^{+++} it was concluded that the Ca-fraction bound to the membrane in control preparations decreased in the presence of 1 mM La^{+++} very significantly (from about 0.8 mM to 0.1 mM per gram wet muscle). The results presented above may prove that

1. a membrane calcium fraction being in equilibrium with the calcium content of the interstitial fluid plays an important part in the electro-mechanical coupling of the muscle and, that

2. La^{+++} can replace most of the membrane calcium and, in this way, interfere with its function.

Autoradiographic Investigation on the Intrafibrillar Localization of Ca with Electron Microscope

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In the course of examinations of the localization of inorganic elements in striated muscle the distribution of ^{45}Ca introduced into the muscle fibril by incubation was examined. It was another aim of the experiments to investigate the change caused by indirect stimulation in the distribution of ^{45}Ca in the muscle contracting well-visibly.

It was found that 70 per cent of the grains originating from the exchanged ^{45}Ca was located over the A-band of the muscle fibril. The great majority of the grains found over the I-band was located in the environment of the Z-line, and the centre of 10 per cent of the grains was exactly on the Z-line.

This percentage distribution of grains did not change upon the effect of stimulation. But a significant alteration was observed in the distribution of grains within the A-bands inasmuch as the number of grains increased significantly near the place where A and I bands join with each other.

Current—Voltage Characteristic of Frog Muscle Fibre Investigated as a Function of Temperature

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A direct method has been developed in this laboratory to determine the current — voltage characteristic. This afforded a possibility to investigate the semiconductor features of muscle in a much more exact way than it had been possible previously.

Subjects of our experiments were frog sartorius muscle fibres, and glass microelectrodes were used for recording. The voltage for measuring the characteristic was produced by a special generator the output signal of which had a shape of triangle and the output resistance of which was relatively small. The recorded characteristic was, in fact, the resultant of that of the microelectrode and of the fibre.

Therefore the voltage — current characteristic of the microelectrode was determined in every case, and the measurement was only performed if the characteristic of the applied electrode was linear.

The current — voltage characteristic of a fibre was calculated on the basis of the two curves of the recording, i.e., the own characteristic of the microelectrode and the resultant of both the electrode and fibre characteristics.

The influence of temperature was investigated over the range of characteristic in which the impaled microelectrode was negative in comparison with the indifference electrode.

Extra-thin Filaments of Striated Muscle

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The wing-muscle of the honey-bee (Apis mellifica) was examined with electron microscope. The muscle was cooled to 0°C before preparation. As a consequence of cooling, the muscle shortened significantly. The muscle was stretched in this state. It has been found that the fibrils are composed of three kinds of filament sets consisting of thick, thin, and extra-thin longitudinal filaments.

The extra-thin filaments were found in the I-band, they connected the thick filaments with the Z-line. The extra-thin filaments were 20 to 30 Å in thickness. A periodicity of about 400 – 450 Å could be seen in these filaments. In some places extensions extruded out of the extra-thin filaments which could transversally connect two extra-thin filaments each.

Study of Conformational Changes in Muscle Tissue by Epr

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The McConnell spin label epr technique was used in order to study the conformational changes of muscle proteins in surviving fibre bundles and glycerinated muscle fibres prepared from frog sartorius and rabbit psoas.

The preparations were incubated in Ringer solution containing 10^{-4} M/litre 2,2,6,6-tetramethylpiperidine-1-oxyl-iodoacetamide spin label at 4°C for 24 hours. A detailed analysis of the epr spectra recorded at room temperature indicates that the spectrum consists of two species of superimposed spectra. The spectra were calculated by using the rotational correlation time and asymmetric parameter.

Denaturation evoked by urea and guanidine hydrochloride changed the epr spectrum. These changes signify an increase in the average motion of the spin label. According to the experiments the urea-induced denaturation is reversible, but the denaturation by guanidine hydrochloride shows some irreversibility.

About the Transversal Structure of Myofibrils

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Fibril of honey-bee wing muscle was stretched transversally with a micromanipulator. The stretched range was examined with phase-contrast and polarization microscope. From the fact that the Z-discs stretched in transversal direction, continuously but the filaments were divided to bundles, the conclusion was drawn that, between the myofilaments, a transversal structure can be found which connects these filaments to each other.

Muscle Crystallization and Water Binding

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Crystallization and water binding, respectively, of frog muscle were examined under the effect of stretching. It was the working hypothesis that stretched muscle crystallizes and — as a result of crystallization — its water binding becomes different from the water-binding of non-stretched muscles. The degree of water-binding can be demonstrated with different methods. In the present experiments, the method of freezing was used.

A New Type of Microcalorimeter for the Investigation of Heat Production of Muscle

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Energetics of muscular activity is an important part of muscular investigation and the microcalorimetrical method is a very good tool for this purpose.

We have developed a new type of microcalorimeter based on the results of our institute concerning muscular heat production.

The more important parts of our system are as follows:

1. The thermopile is a so-called "coiled-coil" copper constantan thermopile. We have got the idea for preparation from C. Kitzinger's and H. Benzinger's work. Our thermopile has about 6000 thermoelements, and its reference and acting ends extend over an area of approximately 50 cm^2 each. We have prepared the thermoelements by the copper-galvanization of the constantan, coiled on a polyethylene tube 3 mm in diameter.

2. Stabilized water-bath, with the help of which we can measure within temperature ranges from $-10°C$ to $+30°C$.

3. Vacuum thermoinsulator for the elimination of thermal disturbances.

Power and displacement transducers and a programmable electromagnet are further parts of the system. The microcalorimeter enables one to measure the heat production of various types of muscular activity.

Plasticity of Muscle during Rest and Contraction

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It is known from our previous work that the elastic behaviour of the active muscle is significantly different from that in a resting state. Our aim was to determine the characteristics of elastic and viscous features in different states of the muscle. For this purpose a dynamic method of examination was developed. A new system of mechano-electric measuring transformer was constructed; its effective mass, its friction occurring during motion and its mechanic power adopted from the system to be measured, are negligible. It has a characteristic curve with a wide-range linearity, and its high sensitivity enables measurements of small shifts.

In our experiments the amplitude and the angle of phase of sinusoidal change of length were measured by stimulating the sartorius muscle of frog (*Rana esculenta*) for longitudinal vibration in passive and tetanic states, respectively. From the data obtained at different frequencies of stimulation a curve of resonance was constructed from the analysis of which the elastic constants could be calculated. The degree of elastic parameters and the degree of energy-dissipation obtained in resting state and in contraction, respectively, are significantly different from each other, from which we can conclude the crystallization of the tetanically stretching muscle.

2. Subject: *Biophysical Basis of Regulation in Biological Systems*

Dielectric Dispersion Measurements on the Dimeric and Monomeric Forms of Phosphorylase *b*

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Rabbit skeletal muscle phosphorylase *b* was investigated by dielectric dispersion method in different media.

Comparison of the calculated and measured relaxation times showed molecular diameters higher than published earlier.

Estimating the thickness of hydrate shell of protein to be 4 Å we find a good agreement between the calculated and measured relaxation times.

On the basis of these calculations and measurements the molecular dimensions of hydrated phosphorylase *b* were estimated.

The molecular diameters appeared to be $119 \times 63 \times 73$ Å.

In the presence of p-chloromercuribenzoic acid (PCMB) the phosphorylase *b* dissociated into subunits.

Dispersion measurements showed that the volume of monomeric phosphorylase *b* (in the presence of PCMB) was smaller than the half volume of the dimeric form.

This result suggests that the dimeric phosphorylase *b* molecule has an "intermediate" volume.

Examination of Iron-Metabolism by Whole-body Counting in Mice

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The stimulatory or inhibitory effect on iron metabolism of plasma of persons suffering from different haematological diseases was studied in mice.

The experiments were performed with a one-crystal whole-body counting measuring set with decreased background.

The changes of iron metabolism were traced by $^{59}FeCl_3$ diluted with physiological saline and administered to the experimental animals at the beginning of different plasma treatments.

The biological half-life of each group was calculated with the aid of the effective half-life obtained from the decrease of the effective impulse of different groups.

It was experienced that certain plasmas stimulated while others inhibited the iron metabolism as compared with the plasma of normal haemo-state. From the changes of iron metabolism the authors concluded the effect on blood production of the animals.

Transfer of Electronic Excitation Energy from Lutein to Chlorophyll-*a* in Detergent Micelles

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Mixed solutions of lutein and chlorophyll-*a* with Triton X-100 detergent were used for studying the transfer. Absorption, fluorescence and fluorescence excitation spectra were determined at different detergent concentrations and temperatures. From these characteristics the most important features of lutein → chlorophyll-*a* energy transfer could be established. The detergent micelles play a double role: they ensure a high local concentration of the pigments and thus an effective lutein → chlorophyll-*a* energy transfer; furthermore, they serve as a suitable matrix for ordered pigment arrangement.

The results suggest that the detergent solutions containing carotinoid and chlorophyll molecules can be regarded as proper model systems for studying the regulation of primary processes of photosynthesis and that of the transfer of electronic excitation energy in particular.

Interaction between Chlorophyll-*a* Molecules in Detergent Micelles

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The chlorophyll-detergent model used for the study of the primary processes of photosynthesis is based on the fact that the detergent molecules form micelles at a certain detergent concentration, the so-called critical micelle concentration (CMC). The chlorophyll molecules are incorporated into the micelles and this process can be traced by measuring the light absorption and optical activity of chlorophyll-*a*. These characteristics were determined as functions of the time and the concentration of Triton X-100 at 30°C.

The peaks of the absorption spectra in acetone at 430 and 662 nm are shifted to 435 and 668 nm, respectively, in the detergent solution. At concentrations below the CMC ($4 \cdot 10^{-4}$ M Triton) the absorption spectra practically do not change, while above the CMC the intensity of the 670 nm band decreases and a new band increasing in time appears at 745 nm.

The CD spectra arising from chlorophyll–chlorophyll interaction are the results of a superposition of several forms of chlorophyll-*a*. The initial peak at 662 nm shows the optical activity of the monomer, while the developing peaks at 638, 713 and 750 nm are attributed to the presence of dimeric and highly aggregated chlorophyll, respectively. Above the CMC the intensities of CD spectra significantly increase and a change in the distribution of intensities can be observed.

All these data may be interpreted as the result of changes in the state of chlorophyll-*a* incorporated into the detergent micelles.

Basis of Reference of the Photo-Synthetic Energy-Utilization of Plants and Plant Stands

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In calculating photosynthetic energy-utilization the most important thing is to determine the light energy which, of the whole radiation, forms the energy basis of the photosynthesis. It depends significantly on the shading and spatial position of the leaves beside the structure of tissues of the photosynthetic pigments and the leaf.

Not considering the two latter factors, in tomatoes grown under conditioned circumstances at an illumination of 8.500 Lux, the utilization of energy is 2.4 per cent if we take the whole light energy as a starting point, and 4.8 per cent if we consider the in vivo absorption of the leaf and the light absorption of photosynthetic pigments.

In the case of plant stands the reference can also be done for areas of growth, because it is very difficult to determine the surface of the leaf. The average of the energy measured in the stand can be considered as the light energy at disposal. Reference to the surface of leaves, which does not cause a problem in certain plants in autoecological examinations, is more reasonable than this.

Because of the absorption of photosynthetic pigments, also the spectral composition of the light forming the energy basis of photosynthesis changes in relation to the incident energy, inasmuch as the rate of energy falling within the wave-range of violet and blue increases, while the rest decreases or changes but slightly.

Application of Several Compartment Models for Interpreting the Iodine Kinetics of the Organism

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Three and seven compartment models were made for the examination of the iodine kinetics of the organism. The constant of iodine kinetics characteristic of the processes were determined by solving the differential equation system belonging to the models. In a three compartment model our calculations were performed by solving the system of equations and, in a seven compartment model, by curve-fitting with an analogous computer. The velocity constants obtained in two different ways show agreement within the limits of error.

In the case of adopting appropriate experimental curves our method seems suitable for the quantitative evaluation of spontaneously occurring disorder of iodine metabolism, and for characterizing the effect of certain agents on the activity of the thyroid gland.

Investigation of the Factors Influencing the Phage Development in T7 Phage–*E. coli* System

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A model has been constructed which characterizes the phage development, and can be used in exactly determined conditions only. The model was compared with previous experimental data obtained in T7 phage–*E. coli* system (modified one step experiment). The con-

ditions of experiments, required to use this model, were ensured in a chemostate. Such conditions were: a statistical distribution of the age of individual germs in bacterial culture, a constant growth-rate of bacteria during the experiments, and an infection of low multiplicity.

The present paper describes the results concerning the changes of number of free phages. From these data the mean value of the burst size, the duration of the burst time, the scattering of distribution, as well as the adsorption constant can be concluded. All these are determined by the generation time (dilution rate) of the bacteria kept at a constant value during the experiment.

Repetitive Response of Sensitized and Illuminated Frog Sciatic Nerve

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As it was pointed out in our earlier photodynamic experiments, action potentials will be generated by the effect of intense illumination in frog nerves stained by dilute eosin. However, these action potentials occurred at random and were, therefore, recordable by chance only.

In the present paper a photodynamic phenomenon will be described which is very well reproducible and suitable to study some details of the photodynamic action. Dissected frog sciatic nerves were stained by eosin dissolved in normal Ringer solution (concentration of eosin ranged from 10^{-4} to 10^{-8} g/ml). If the stained and illuminated nerve was stimulated by a single electric pulse a burst of spikes was observed instead of the expected single action potential. A latency time of one minute or some more preceded the phenomenon. Its duration was inversely proportional to the concentration of the sensitizer and to the intensity of illumination. After switching off the illumination the repetitive response persisted for a while, but later it disappeared, and one single spike was evoked by one electric stimulus; then the experiment could be repeated once again with the same nerve.

The results are interpreted as electronic processes of the excitable tissues.

Repetitive Excitation of the Nerve-muscle Preparation

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Continuing previous investigations from which the conclusion was drawn that the motor nerve also had an ability to respond to a single electrical stimulus with repetitive excitation, in the present series of experiments the author has examined the interrelationship between the stimulus intensity and the repetitive response of the nerve and the muscle.

The experiments were performed on frog nerve-muscle preparations (sciatic nerve and gastrocnemius muscle). The proximal part of the nerve which had been soaked in Ringer solution containing glycerin was stimulated with square-wave impulses 0.1 ms duration and 0.2—2.0 V amplitude. The action potentials of both the nerve and muscle as well as the muscle contractions were recorded. The results showed that a rise of the stimulus intensity brought about an increase in the electrical activity of the nerve and the muscle while the mechanical response of the muscle was a tetanic contraction.

The correlation between the stimulus intensity and the excitatory activity can be related to a coding process taking place in transformation of stimulus into nerve excitation also in the case of the motor nerve.

Causal and Teleological Models of a Biological Automatism with Periodic Operation

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When interpreting a natural phenomenon in terms of causal relationship the phenomenon is broken down into events where each of them is directly to determine the next one in sequence. When approaching the phenomenon on a teleological basis, however, the whole phenomenon is considered to be a single entity. These two different interpretations may make use of different laws and principles for describing the phenomenon. Similarly, the mathematical tools applied may well vary but they are strictly equivalent. (See: J. Neumann, The Unity of Knowledge, L. Leavy, Doubleday, NY. 1955.)

It has been our ambition to investigate whether the above principle can be applied in analysing a particular biological phenomenon by using the above dual discussion throughout. As an obvious "field" for testing our ideas the endocrine control systems were chosen. In some earlier publications the quantitative model of the female endocrine control system has been already described in terms of strict causal relationship. The model has been proved to be right in computer simulation. In line with the above aim, as a first step towards creating the teleological model of the same biological phenomenon, a graphical method for displaying the periodic nature of the endocrine process as well as the selection of mathematical variables for describing the crucial factors of the biological process have been developed.

3. Subject: *Physico-chemical Basis and Energetics of Membrane Activity*

New Data on the Ion Mechanisms of Potential Generation

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In the ion theory of nerve excitability and spike generation formulated by Hodgkin and Huxley the inequal distribution of ions inside and outside of the cell and the passive properties of the membrane are considered as main factors of potential differences. However, data are accumulating showing that, in some cases, active membrane processes, e.g. the electrogenic sodium pump contributes to the membrane potential (Kostyuk, 1972; Thomas, 1972). This pump is sensitive to ouabain, it can be stimulated by increasing the intracellular sodium or the extracellular potassium concentration and can be inhibited by low temperature or low external potassium (Jansen and Nicholls, 1973; De Weer and Geduldig, 1973; Carpenteri 1973). The possible role of electrogenic Na-pump in the generation of burst activity (Salánki et al., 1973) is discussed.

New results suggest that beside sodium also calcium ions take part as current carriers in the spike generation of some neurons (Hirst and Spence, 1973). Because of the membrane stabilizing role of Ca thorough experiments are needed to prove this possibility. Recent data of Stinnakre and Tauc (1973) using the photoprotein, aequorin directly demonstrated the Ca-influx into neuron during spike generation. Similar mechanism can be supposed in those Lymnaea neurones, where spikes remain intact in Na-free solution for a long period of time, but they are absent in Ca-free medium (Kiss and Salánki, 1973).

The different chemical sensitivity of the neighbouring neurons (Kiss and Salánki, 1971) and further, the ionic differences found between somatic and axonal potentials in the same neuron (Wald, 1973) show that a simple application of the original or modified (Hodgkin and Keynes) ion theory cannot be suitable for explaining many of the recent experimental data.

Effect of Low Concentration of Heavy Water on the Activity of Nerve Cells of *H. pomatia*

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The effect of 13 per cent heavy water (D_2O) on "dumb" and spontaneous cells of sub-oesophageal ganglions of *H. pomatia* was examined.

A decrease in the stimulus threshold was experienced in dumb cells; this decrease also caused the appearance of a series of discharges in several cases.

In spontaneous cells a 20 to 40 per cent increase of the discharge frequency was observed upon the effect of D_2O. The effects appeared with a short latency (30 to 90 sec) after the introduction of D_2O, and they faded out within 4 to 5 minutes.

The authors discuss their results within the frame of the polyelectrolyte theory of bioelectrogenesis.

The Effect of Heavy Water on the Activity of Frog Heart

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Straub- frog heart (*Rana esculenta*) preparations were used in the experiments. The hearts had been washed with K-free Ringer solution several times until they stopped. A half of the hearts (control) was further treated with K-free Ringer solution, while the remaining part with K-free Ringer solution containing 1 and 5 per cent of D_2O, respectively. The solution containing 1 per cent of heavy water failed to cause any essential difference between the function of treated and control hearts. However, the increase of D_2O content affected the function of the heart in a positive way. The experiments were evaluated with χ^2 test. A significant difference was found between hearts treated with K-free Ringer solution containing 5 per cent of heavy water and with K-free Ringer solution containing normal water ($p = 0.001$).

Osmosis of Water into Heavy Water

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The first step to the exact knowledge of biological transport processes is to get acquainted with the quantitative connections of transport processes in simple models and the molecular–submolecular mechanism of these processes. The vapour pressure theory of osmosis is supported by our data according to which pure water having a higher vapour-pressure flows

into concentrate heavy water having lower vapour pressure through a $Cu_2Fe(CN)_6$ membrane at room temperature even against a pressure of 1.5 atm.; i.e., osmosis is possible without the presence of solved crystallides or colloides. But the further data indicate that the applicability of the theories is limited. E.g. the concentrate heavy water of lower vapour pressure flows into the NaCl solution containing normal water and having higher vapour-pressure. Also the thermodinamical–phenomenological description is a problem because our system has a rectifying property towards hydraulic water flow, and, thus, linearity practically does not exist.

Connection between the Second Messenger System and Electrogenesis in the Membrane of Insect Hearts

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Intracellularly recorded resting and action potentials of heart muscle cells were examined with the aid of microelectrodes in the heart of *Locusta migratoria migratorioides*. The depolarization of the membrane of heart muscle cells and the increase of the AP frequency in spontaneously active hearts, by cyclic 3′,5′-Amp (CAMP) as well as dibutyryl 3′,5′-AMP (db-CAMP) were stated. At the time of frequency increases the duration of AP increases as a result of prolongation of the plateau-phase. db-CAMP has a more expressed plateau-phase increasing effect. The prolonged effect of the above substances brings to a block of the potential generation this can be easily restored by rinsing. Theophylline, caffeine and NaF also increase the frequency of AP, but to a smaller degree only. The above effects are modulated by deionizations.

The contractility of the heart of Locusta is also increased by CAMP, db-CAMP as well as theophylline, caffeine and NaF, among which db-CAMP is the most effective agent.

Effect of Local Anaesthetics on Soma Membrane of Giant Neurons

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The effect of lidocaine and the substance RG—1812 was examined on the spontaneous activity and chemical sensitivity of giant neurons of *Lymnaea stagnalis*. It is characteristic of both drugs that they inhibit the generation of action potential and prevent the stimulating effect of ACh. The two different effects can be supposed to be manifest on separate receptors, as their time- and concentration-dependence do not agree.

During the blocking of action potential the rising and falling phase are equally damaged, which allows us to suppose the simultaneous block of Na and K channels.

The effect of the substance RG—1812 differs from that of lidocaine only quantitatively: it inhibits the formation of spontaneous potential more weakly, to a lesser degree but, at the same time, it has a stronger effect on ACh sensitivity.

Effect of Transition of Temperature on the Parameters of Activity of L.Br-Type Cells of *Helix pomatia*

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RPal cells described in the central nervous system of *Helix pomatia* (Sakharov and Salánki, 1970) show a characteristic Br-type activity (Arvanitaki and Chalazonitis, 1955). According to earlier investigations they can be considered as intraganglionic cells (Salánki et al., 1972), and can be supposed to play an important role also in the regulation of other cells.

The activity of these cells strongly depends on the temperature. The Br-like activity pattern only occurs within the given temperature ranges (Salánki et al., 1973).

According to present examinations the transient responses caused by the change of temperature can be separated from the absolute temperature dependence of the activity. This shows that the parameters of the activity of the cell not only depend on the stationary temperature, but also on the velocity of the temperature change.

Stochastic Model of the Alkali Ion-Transport of Plasma-Membranes

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A possible way for the quantitative description of alkali ion-transport is presented here.

The functional model treats the active and passive membrane transport uniformly. The necessary mathematical–physical bases are supplied by the theory of stochastic processes.

It has been supposed that the defects occurring necessarily because of thermodynamical causes in the phospholipid phase of the membrane serve as places for ion transmission. We further suppose the energy of the reaction activated by the following ATPase reaction

$$\text{ATP} + \text{Enzyme} \xrightarrow[\text{Na}]{} \text{ADP} + \text{P} \sim \text{Enzyme}$$

to bring about a change perpendicular to the surface of the membrane.

Our model was fit into the results of isotope-kinetical examinations with the aid of computer evaluation. On the basis of the parameters obtained we can conclude to the strength of the ion–membrane interaction, as well as to the mode of action of the drugs applied.

Model Fitting of Transportkinetic Data

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In the study of the transportkinetic processes we usually start from differential equations. In some cases the solution of a system of the differential equations cannot be expressed in terms of known functions, e.g. exponentials. Therefore, in the analysis of the transportkinetic data, it is of interest to fit the differential equations directly. We employed a least square procedure, starting with initial estimates for parameters in the fitting. To minimize the sum of squares we used Newton—Raphson gradient method and Nelder—Mead simplex procedure. By means of the FORTRAN programs the uniqueness and the consistency of

the ion transport model — given by I. Sugár and S. Györgyi — have been examined. The model was consistent (examined by F-test to compare with a suitable polinomial regression), but it was ambiguous when we used only the curve of ^{42}K isotope decrease in plasma.

Electrochemical Oscillations on Bimolecular Lipid Membranes

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Pant and Rosenberg reported sustained, coupled electrical and mechanical oscillations on bimolecular lipid membranes (BLM) separating aqueous solutions of $K_3[Fe(CN)_6]$ and KI. The phenomena can be characterized as follows:

1. The oscillatory behaviour is membrane-specific.
2. The oscillations are practically undamped.
3. The phenomena are decisively pH-dependent.
4. The amplitude depends on the concentration of KI.
5. The frequency is a function of pH.
6. The oscillations are voltage controlled.

Pant and Rosenberg suggested that a similar or even the same mechanism might be responsible for the oscillations described by Theorell and Franck.

On the basis of more recent investigations it seems to be very plausible that the voltage and current fluctuations are primarily of electronic origin. This interpretation supports some further assumptions of theoretical and practical importance:

1. The iodine-BLM systems are predominantly electronic conductors.
2. The interfaces may behave as semiconductor electrochemical electrodes.
3. Coupled (synchronized) electrochemical electrode reactions occuring at the interfaces are capable of bringing about sustained and temporary processes of regulation and passing of information.
4. Electrochemical deposition on, and liberation from, the membrane surface endows the membrane with dynamic character.
5. The coupled diffusion and phase boundary effects may play an important part in eliciting biological oscillations.

The Effect of Ionic Environment on Electrical Properties of Bimolecular Lipid Membranes

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The step requiring most energy in charge carrier transport through bimolecular lipid membranes (BLM) is the entry of charge carriers from the aqueous phase into the membrane phase. The mechanism and character of entry processes can be investigated by measuring the current–voltage (I—V) characteristics.

Certain electrical processes of 2 : 1 lecithin–cholesterol membranes separating KCl solutions were studied. KCl solutions were found to behave like ohmic injecting electrodes

at low transmembrane potentials, while the character of the I—V curves reminded of space charge limited charge carrier injection at higher potentials. In the low potential range (i.e., $V < 60$ mV) the current is a linear function of the voltage and in the high potential range ($V > 90$ mV) the current is proportional to higher powers of voltage. The intersection of the two linear sections in log-log plot (V_0) seems to be a monotonous function of ionic strength of the bathing solutions and is shifted towards lower potentials with increasing temperature. A possible mechanism of charge carrier injection will be presented.

Dielectric Investigations on Lipid-iodine Systems

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The electrical conductivity of both lipids and artificial bimolecular lipid membranes drastically increases in the presence of iodine. The iodine forms charge transfer complexes with lipids (lecithin, oxidized cholesterol etc.). Neither the nature of these charge transfer complexes nor the mechanism of electrical conduction (ionic or electronic conduction; hopping or tunnelling etc.) are fully elucidated.

In order to clarify the conductivity mechanism dielectric investigations were carried out on lipid–iodine complexes in the frequency range of 100 hZ—1 MHz. On the basis of measurements made on hydrocarbon solutions of these complexes it can be concluded that no ion-pair formation occurs at low electric field strength in the solutions. The measurements made on solid samples confirm the conductivity-increasing effect of iodine and render probable the electronic nature of conductivity. It can be supposed that the Maxwell—Wagner polarization may be responsible for the frequency dependence of dielectric constant, and the high conductivity of the solid complexes may be attributed to this effect. The hydration state plays an important role in the electric and dielectric properties of the complexes investigated.

Kinetic Studies on Chlorophyll-containing Membrane-forming Extracts

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In aliphatic hydrocarbon solution of spinach leaf extract containing chlorophyll, as well as in thin membranes formed from such extract, changes can be observed in the absorption spectrum, which are usually attributed to the appearance of chlorophyll in a crystalline form.

The spectral changes can be hindered by adding certain amounts of lipids to the extract, or by diluting it.

The time curve of the changes renders it probable that they are the resultant of two processes; the faster one is localized in the phase boundaries, and the slower one takes place in the bulk phase.

Comparative Studies on Lipid Membranes Containing Photosynthetic Pigments

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The artificial lipid membranes containing chlorophyll pigments (Chl-BLM) are useful model systems for studying the charge transfer processes in photosynthetic membranes. Chl-BLMs were prepared from spinach extracts. The composition of the membrane-forming material was analyzed both chromatographically and photometrically. The membranes made from different extracts were compared by absorption and electrical measurements. From the absorption measurements it seems probable that at low pigment–lipid ratio (0.3) the chlorophylls are in monomeric or dimeric form, while at high ratio (2.0) they are in an ordered ("crystalline") state. Practically no difference could be observed in the electrical properties of membranes of different pigment–lipid ratios.

Diffusion of Alkali Ions through Lipid Membranes

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In the authors' previous experiments the effect of antibiotics on the ion-transport of *E. coli B.* was examined. But the living cell is a very complex system, it is difficult to follow the processes going on in it. The liquid crystal system prepared from phospholipids, the so-called "liposome," has a great resemblance to biological membranes in some of its characteristics. At the same time it has a more simple structure. That is why it is an appropriate model for the examination of transport processes.

In the present experiments the effect of polymyxin, an antibiotic affecting penetration was studied on ^{24}Na, ^{42}K and ^{86}Rb transport in such a model membrane. A mathematical model was used for the evaluation of the experiments, and the constant of time of penetration was calculated in each case. No significant difference was found in the penetration of the 3 ions. Upon the effect of polymyxin the permeability of liposome increased for ^{24}Na and ^{42}K and did not change for Rb.

Effect of Ionophorous Antibiotics on the Cation Transport of Human Erythrocytes

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Macrocyclic and aliphatic polypeptide antibiotics enhance the monovalent cation transport of cell membranes. In the present experiments the effect of six various antibiotics was studied on the ion transport of human erythrocytes. The effectivity of the tested antibiotics was as follows: Nigericin > Valinomycin > Gramicidin > Candicidin > Nystatin > Bacitracin.

A significant difference was found in the ion selectivity of the various antibiotics. The increase in the transport rate was unidirectional in every case: only the downhill transport rate was affected.

The results suggest that the antibiotics fulfill the role of a special carrier.

Data on the Problem of the Relationship between Energetics of Foetal Membrane Transport Processes and Placentar Oestrogens

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From the maternal circulation nutritive substances gain access to the foetal circulation through the chorionic villi developed from the foetal membrane. The membrane plays an active role in the transport of the essential substances like amino acids, inorganic phosphate, iron, calcium etc. A long-lasting decrease in the transport leads to "malnutrition" while its acute diminution, the "placentar insufficiency," may cause intrauterine death.

Earlier data of the literature suggested that the oestron and oestradiol produced in the placenta influenced the ATP synthesis of the placenta in a positive manner via a specific oestrogen-dependent iso-citric acid dehydrogenase (ICDH) having a NAD hydrogen acceptor instead of the usual NADP. The author examined the oestrogen-dependent ICDH activity of placentae obtained from normal birth or interruptions performed on the basis of social indications and compared it with the activity of ICDH, MDH and LDH. The oestrogen-dependent ICDH constituted merely 5—10 per cent of the ICDH activity responding to NADP, and it was more than 100 times less than the MDH or LDH activity.

The Effect of TRITON X-100 on the Functional Properties of Sarcoplasmic Reticular Fraction

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The sarcoplasmic reticular fraction (SRF) used in the experiments was prepared from the white muscle of the cat-fish (*Ameiurus nebulosus*). The myofibrillar protein content was removed by extraction with 0.6 M KCl solution. Subsequently, SRF was extracted with Triton X-100 applied in various detergent: protein ratios. It was found that with a detergent: protein ratio of 1 : 1, both the cholinesterase and the ATPase activity of SRF increased very markedly (to about 300 per cent) while the Ca uptake ceased. At the same time the cholinesterase component of SRF was solubilized almost completely, while its membrane ATPase remained structure-bound. If the detergent : protein ratio was raised to 2.5 : 1 also most of membrane ATPase became solubilized. This solubilization of ATPase was accompanied with a reduction of its enzymic activity. The cholinesterase fraction of SRF isolated after TRITON X-100 treatment proved to be homogeneous in sedimentation and diffusion studies ($S_{20,w}$: 1.84S; $D_{20,w}$: 2.9×10^{-7} cm^2 sec^{-1}; f/f_o: 3; molecular weight: 55.000). The structural changes of SRF produced by detergent treatment were also investigated in electron microscopic studies.

Problems of the Ion Absorption Research in the Higher Plants

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The investigations of the absorption mechanisms in higher plants do not keep pace with the results obtained in other living organisms. Behind the subjective reasons we have to find the real problems of the transport in plant cells.

The main question is the energy supply of the plasmalemma for the active absorption. The utilization of ATP is doubtful because at present nobody can be sure about the presence of ATPase in the transport barrier of plant cells.

The active transport was proved in indirect way by the low concentration range for K^+ and Br^- uptake. The inhibition between K : Rb and Br : Cl is competitive. The characteristics of concentration curves remain unaltered in the presence of Ca ions or 6 hours after the excision, in spite of the significant increase in the rate of ion uptake after these different treatments.

However, it is remarkable that the ion content of the cells reduces the ion uptake and ion exchange in different degrees. The influx of ^{42}K at low external concentrations is nearly fully inhibited by cells preloaded with unlabelled potassium ions.

The absorption rate of ions did not remain constant after the excision of wheat roots. Shaking the excised roots in $CaSO_4$ solution causes the ion uptake capacity to increase to 2–3 fold of the original values. This so-called isolation effect is more pronounced at the apex of the roots than at the basis. The isolation effect can be inhibited by fluoroacetic acid and by cycloheximide.

The preloading and isolation effects make it more difficult to obtain the efflux values of wheat root cells.

We can form a true conception about the ion transport if the technical development allows manyfold and simultaneous measurements of the ion out- and inflow through the plasmalemma.

Effect of Light $\rightleftharpoons$ Dark Transition Treatments on the Uptake of ^{42}K by *Scenedesmus obtusiusculus*

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The mechanism of the cation and anion transport was studied in sychronous culture of the unicellular alga *Scenedesmus obtusiusculus* CHOD. The uptake difference between the light and dark periods called for the problem, how light and dark or dark and light treatments affected ^{42}K uptake.

According to the results light stimulated the ^{42}K uptake of the cells grown both in dark and light. The rate of ^{42}K influx reached a maximum within a short period of time (2–4 minutes) under the effect of dark and light or light and dark pretreatments. Further light or dark pretreatments did not augment the amount of ^{42}K taken up in a unit of time (10–30 minutes), but caused a decreasing tendency from the 5th minute of pretreatment.

It can be assumed from the experiments that the initial rapid increase in the ^{42}K influx rate is not the consequence of light pretreatments, for the process can also be elicited by pretreatments only in dark. On the basis of experiments performed in different variations it can be concluded that it is a rapid shock-like effect, caused neither by the transfer of cells into a "Ca + Mg-solution" from the medium, nor by a change in pH.

4. Subject: *Biophysical Basis of Effect of Physical Factors on Biological Systems at Molecular and Cellular Level*

Examination of the Mechanism of Radiation Effects by Grid Irradiations

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Little is known about the mechanism of radiation effects. Therefore a special study was started concerning the phenomena taking place in irradiations through a grid for which no explanation could be found so far. To be precise: the role of the regeneration processes starting from the covered volume parts in the reduction of radiation effects developed in the volume elements irradiated through the apertures of the grid was examined, and the problem was investigated whether such radiation effects indicative of the presence of macroscopic changes of place do or do not exist at all.

Barley seeds previously treated with water, sown in an 1 cm square mesh and standing vertically, were irradiated with various doses of ^{60}Co gamma rays, partly in completely dry soil, partly in soil saturated with water immediately before the irradiation. In the series irradiated in dry soil and watered afterwards the coming up of the sowings precisely corresponded to the projection of the grid, that is to say, the barley seeds under the apertures did not come up at all, whereas in the covered parts they came up without failure just like did the controls, and they started to grow.

After irradiation in a watery soil the picture of the sowings was quite different. Seeds that had come up were also found in the uncovered parts, while significant damages were experienced also in the covered parts, a finding pointing to the significant role of water, and/or to some sort of macroscopic motion.

Some Data on the Changes in the Haematological Picture of Normal and Radiation-damaged, Di-Adreson F-treated Mice I.II.

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The two lymphocyte subpopulations are known to differ in their sensitivities to PHA, X-irradiation and cortisone. To present knowledge, the cortisone derivatives act on the small lymphocytes of a short life span, both at the cellular and the humoral level. After low doses this effect only shows in immunosuppression, after higher doses also in the degradation of lymphocytes. An adequate PHA dose increases the count of lymphocytes, most markedly by the 3rd to 4th days after the treatment.

Female F_1 hybrid (CBA/BALBc) mice of 20 to 22 g were used. Di-Adreson F applied in these experiments is by 3 to 5 times more effective than cortisone acetate.

The joint effects of PHA and Di-Adreson F on the lymphoid system and on lymphoid regeneration in 350 R X-irradiated mice, respectively, were studied.

The results suggest that the action of cortisone might be reduced by PHA.

The Role of Circulating Haemopoietic Primordial Cells in the Regeneration of Radiation-damaged Bone Marrow

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The experiments were designed to elucidate what role the haemopoietic primordial cells released from the shielded region of the body, play in the regeneration of radiation damage in partially irradiated mice. Mice with one tibia irradiated with 650 R collimated X-rays were used throughout the experiments. Regeneration rate was measured in the irradiated tibia. In some experimental groups the release of primordial cells from the shielded body region was reduced, in other experimental groups stimulated by appropriate methods.

A correlation was found to exist between the number of primordial cells circulating in the peripheral blood and the rate of regeneration. In the groups with reduced circulating haemopoietic primordial cell count the regeneration amounted but to 5 to 15 per cent during the experimental period, while in the groups where the circulating haemopoietic primordial cell count had been appreciably increased, a 50 per cent regeneration could be measured.

Change of the Osmotic Resistance of Erythrocytes upon the Effect of X-ray and Radiation Protective Compounds

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The osmotic resistance of erythrocytes was examined after in vitro and in vivo irradiation with or without the application of chemical radiation protectives. The osmotic resistance was found to decrease upon the effect of 1000 R irradiation of the mouse; AET given alone, or combined with cysteine, moderated this decrease. AET also increased the resistance of non-irradiated normal erythrocytes. The result of the authors' previous experiments is corroborated by the present finding that the radiation protective action of radioprotectors containing SH involves an additional effect which moderates the increased permeability of tissue due the effect of ionizing radiation.

Repair Mechanism of the Erythropoietic System after Ionizing Radiation of Different Quality and the Effect of Radioprotective Compounds

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Upon the effect of electromagnetic (X-ray and ^{60}Co-gamma) as well as corpuscular (reactor neutron + gamma) irradiation of different quality, the erythropoiesis will be damaged depending on the dose, time and quality of irradiation. The degree of damage and the dynamics of repair were determined by a ^{59}Fe-utilization test after single and repeated irradiations with

different doses. A significant difference was found between the kinetics of intracellular and repopulation repair upon the effect of sulphur-containing radioprotective compounds, after those of the indolylalkylamine type, as well as after their combined administration. A close relation was found between the effectiveness of protecting action and the LET value of irradiation, which could be brought into correlation with the repair mechanism.

Effect of Ionizing Radiation on the Glycoprotein Synthesis of Eucaryota Cells

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Radioactive glucosamine uptake of in vitro cultured primary chicken fibroblast cells (PCFS) was examined in control and irradiated cultures. The method of light microscopic autoradiography was used for determining the degree of incorporation.

Cells in interphase took up the precursor in increasing amount, depending on the time of incubation. However, two stages could be distinguished in the speed of incorporation. On the basis of data obtained the glycoprotein synthesis can be supposed to stop or to decrease about 20—30 min before mitosis, and a part of the glycoprotein formed in the interphase will also be decomposed. Beside reorganisation of Golgi complex the incorporation increases at the end of the anaphase and especially in the telephase.

Some 40 min after the irradiation with 1000 rad a significant decrease of mitotic index was observed, indicative of a partial block ensuing at the end of G_2 phase. Swollen "foamy" cytoplasma and, frequently, decomposed cell nuclei were observed in the majority of the irradiated cells. The uptake of labelled precursor into the irradiated cells in interphase did not show any significant difference from the control. According to the data obtained complex glycoprotein synthesis is a rather radiation-resistant process.

Toxicity and Radioprotective Effect of Basic Xantogenic Acid Derivatives in Animal Experiments

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The clinical application of aminoalcylthiols, the most effective radioprotective compounds so far known, is hindered by their several untoward side effects. It is desirable to produce new compounds, which are significantly less toxic but have about the same degree of radioprotective effect. In the present experiments 9 new basic derivatives of xantogenic acid also containing sulphur were prepared, the majority of which satisfies this requirement. Considering toxicity and radiation-protective effect, as tested in white mice, the compounds proved less toxic and had an effectiveness approximating that of AET on an equimolar base. Xantogenates also approximate the effectiveness of AET against the mixed neutrongamma radiation of the atomic reactor, 4 compounds even surpass this. As to stability, toxicity and radioprotective effect, the piperidine-, pyrrolidine- and diethylamine derivatives deserve attention from the point of view of a possible clinical application. The lecture summarizes the toxicity and survival data obtained with this group of compounds, as well as the results of the functional (ECG) examination of the cardiovascular system.

Effects of Different Air-ions on the ECG of Experimental Animals in Irradiated and Normal State

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Air-ions demonstrably influence the function of the heart and circulatory system depending on their concentration. In ECG investigations each animal served as its own control. The duration of air-ion inhalation was 1 hour, the produced ion-concentration was recorded separately in each case before ECG recordings. (A Medicor "Bion-80" apparatus was used for air-ion inhalation, in which the ionizing voltage is 4 kV, and the quotient of unipolarity practically zero.) In other experiments the ECG changes of irradiated mice under the effect of air-ions were recorded. The applied X-ray doses of irradiation were 630 R and 800 R. The changes in the ECG curve experienced upon the effect of air-ions 2 and 24 hours after the irradiation were analyzed.

The Influence of Altering Biological Parameters in the Determination of Internal Doses

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In the determination of the internal doses of radioactive diagnostics the values of accumulation and elimination obtained in animal experiments are usually adopted to human conditions. In the calculation of dose values the distribution of the diagnostics in human organs is supposed to equal that determined in animal experiments.

The internal doses are calculated for the anatomic conditions of "normal" humans weighing 70 kg, both for the critical organs and the gonads.

The lecture treats the magnitude of dose values reaching the critical organs in cases differing anatomically from standard conditions. The values calculated in this way are compared with the doses obtained on the basis of the standard model.

The Role of Errors in Dosimetry in Projecting Biological Experiments

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In course of biological experiments it is either the biological effectivity of an ionizing radiation on some chosen reference radiation (e.g. Co-60 γ-radiation) that is studied or experiments are performed by the same radiation quality to determine the dose reduction factor for some agent. In case of mixed radiation either the RBE value for the radiation as a whole or for some of its components will be determined.

Depending on its objective, the experiment involves different requirements in connection with dosimetric measurements. Generally, the given accuracy of dosimetrical measurements determines how many animals are necessary to perform a certain type of biological experiment.

On the basis of authors' own experiments on CFLP white mice the paper demonstrates that, in the case of various dosimetrical errors, to what extent are survival, spleen weight changes and iron incorporation suitable for determining the RBE value for neutrons and the dose reduction factor of radioprotective compounds in a mixed neutron-γ radiation field.

Study on Nuclear RNA-synthesis after Local X-ray Irradiation on the Liver in Normal and Partially Hepatectomized Rats

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After partial hepatectomy a certain synchronization can be observed in the mitotic cycle of the regenerating liver cell. This permits to intervene by irradiation in a certain chosen phase of the cell cycle. It is known that radiation exerts an intense inhibitory effect on DNA synthesis but the radiation-induced changes in ribonucleoprotein synthesis are less known.

In the present experiments the liver region of the rats was locally irradiated with 50 R, 300 R, 500 R and 900 R, respectively, 6 and 24 hours before the partial hepatectomy. Twenty minutes before decapitation the animals were injected with ^{14}C-orotic acid given intravenously. Nuclei were isolated from the regenerating liver. The ribonucleic acids were extracted from the nuclei by phenol-buffer mixture containing 0.3 per cent sodium dodecyl sulfate, at 60°C. The ^{14}C-labelled nuclear RNAs were separated by sucrose density-gradient centrifugation.

It has been established that 1. irradiation of the liver in normal, non-regenerating animals does not change the rate of nuclear RNA synthesis; 2. partial hepatectomy performed after local X-ray irradiation increases the rate of nuclear RNA synthesis as a function of the dose.

Radiation Sensitivity and Repopulation Kinetics of Circulating Haemopoietic Primordial Cells

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It is known that the regeneration of radiation damaged bone marrow can only start from the surviving haemopoietic primordial cells. That is the reason why the probability of surviving irradiation is proportional to the number of surviving primordial cells. As known from the literature, the primordial cells obtained from various haemopoietic organs (bone marrow, spleen, foetal liver) are different in some of their properties. The present experiments were designed to reveal whether the radiosensitivity and other properties of the haemopoietic primordial cells in the peripheral blood would be identical with those of primordial cells in the bone marrow. As established, the radiosensitivity of circulating primordial cells is lower (D_0: 140R) than the radiosensitivity of the bone marrow primordial cells (D_0: 100R). Furthermore, it has been demonstrated that also some other properties of the primordial cells from these two different sites are different: The turnover rate of peripheral cells as well as the ratio of seeding cells in the case of transplantation are higher than those of the bone marrow cells. From the results the conclusion can be drawn that the primordial cells circulating in the periphery constitute a subpopulation of the bone marrow primordial cells.

100 MHz NMR Investigation of Protein Structure

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The NMR is an excellent direct method for studying the nature of catalytic and regulatory sites of the enzymes. The information obtained with the method may compete with data given by X-ray diffraction and what is more, the enzymes are in natural liquid environment.

The present paper deals with the proton resonance spectra of proteins and small molecules in the presence of proteins as disturbing agents.

By the aid of complete proton magnetic resonance spectra the covalent interaction between N-α-Benzoil-L-Argininamid and trypsin has been studied.

The resonance curves of 5′AMP the allosteric activator of the enzyme phosphorylase-*b*, renders it possible to determine the exact distance between certain protons of the nucleotide and a paramagnetic ion (Mn^{++}) specifically bond to the regulatory site of the enzyme.

The effect of different denaturing agents (temperature, ionizing radiation and SH blocking compounds) was investigated on the NMR spectra of the 5′-AMP and phosphorylase-*b* interaction.

Incidence and Repair of X-ray Induced Single-strand Breaks in DNA of *E. coli* K12 Cells

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The incidence and repair of single-strand breaks in DNA of *E. coli* K12 (AB 2497) rec^+ cells induced by ionizing radiation was studied by means of the technique developed by Mc-Grath and Williams. The method is based on the sedimentation pattern of DNA labelled with ^{3}H-thymidine. It was established that the irradiation with a dose of 15 krads significantly changed the sedimentation pattern of DNA. On the other hand it was also shown that the above bacterial strain had an effective repair system, as the cells repaired the radiation induced single-strand breaks within one hour. On appropriate metabolic state increased, while the well-known repair-inhibitors decreased, the recovery of single-strand breaks. The single-strand breaks in cellular DNA, the repair of breaks and the survival of cells, respectively, are discussed.

Examination of Biological Isotope Effect on Sperms

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Several communications have already appeared from this institute on the isotope separating effect of biological systems. Continuing these experiments the difference in the effect of ^{39}K and ^{41}K isotopes was examined in frog sperms. The motility of isolated, ripe sperms and its change in time were followed in fluid media containing ^{41}KCl in 10, 20, 50 and 100 per cent concentration. Sperms kept in a fluid medium of natural isotope composition containing KCl were used as control. Sperms kept their motility in a fluid medium containing 100 per cent of ^{41}KCl longer than in the control group. The half-life time of moving sperms

was significantly longer in the medium containing ^{41}KCl than in the control. The effect only occurred after 2 or 3 days. No significant difference was observed in the solutions containing 10, 20 and 50 per cent of ^{41}KCl.

Effect of X-ray Irradiation on the Regulation of Tryptophan Pyrrolase of Rat Liver

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The tryptophan pyrrolase activity of rat liver increases after treating the animal with l-tryptophan or glucocorticoids. Glucocorticoids increase the synthesis rate of the enzyme, while l-tryptophan reduces the speed of decomposition of tryptophan pyrrolase in adrenalectomized animals leaving the synthesis untouched.

In the present experiments adrenalectomized rats were treated with l-tryptophan (0.25—1 g/kg) and prednisolone (25 mg/kg) intraperitoneally. The irradiation (1000 R) began immediately after the introduction of the materials, and lasted 30 min. The animals were killed 4, 6, 8 hours after the treatment, and the activity of tryptophan pyrrolase was determined in the homogenate of the liver. The X-ray irradiation by itself scarcely influenced the activity. It inhibited the increase induced by the hormone by 15 per cent, and that caused by tryptophan by 45 per cent.

As tryptophan increases the activity of tryptophan pyrrolase by reducing the speed of its decomposition by linking to the enzyme, the effect of X-ray irradiation was also examined on purified enzyme in vitro. The purified enzyme of a specific activity of 100 to 120 units was irradiated in a dilute aqueous solution in nitrogen atmosphere. Upon the effect of irradiation the sensitivity of the enzyme to the allosteric activator (examined with alfa-methyltryptophan) became damaged earlier than the catalytic activity of the enzyme. It is supposed that a similar damage may play a part in the in vivo observed sensitivity of the regulation of the enzyme.

Effect of Minute Cobalt Contamination on Radioluminescence

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The scintillations caused by beta radiation become well measurable with photomultiplier when the excised eye lens of the rabbit is kept in physiological NaCl solution, containing 100 to 300 μCi/ml of tritiated water, for about 48 hours. The number of scintillations was markedly decreased by Co^{2+} ions in concentrations from $5 \cdot 10^{-6}$ to 10^{-3} M. In the case of concentrations lower than $5 \cdot 10^{-4}$ M the relationship between the decrease scintillations (y) and the Co^{2+} concentration measured in mol (x), follows the function $y = x^a$ with a good approximation, and the degree of decrease can reach 80 per cent of the number of scintillations measured in the control.

Experiments are in progress to examine the radiobiological bases and possible clinical utilization of this effect in preventing the dimness of the lens sometimes following certain radiotherapeutic treatments.

A Possible Model for Lysogen Induction

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The model previously constructed by the authors for radiation damage of T7 phages was further developed, and used to interpret the lysogen induction evoked by UV light. It has been supposed on the basis of the results of one of the authors (D. N.) that lysogen induction is provoked by pyrimidine-photoproducts (thymine-dimers) in bacterial chromosome, causing a disturbance in the bacterial DNA-synthesis.

The functions of damages depending on radiation dose were calculated with the help of the radiation kinetic model described, also considering the dark reactivation. These calculated data were compared with the experimental ones.

The Effect of N-acetylaethylenimine on T7 Phages

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N-acetylaethylenimine has recently been a very frequently used radiomimetic agent. In the authors present work the in vitro experiments of the literature (Krugljakova' team) were compared with own experiments concerning the inactivation of T7 phages, with the aim to characterize the kinetics of inactivation. The inactivation dose-effect curves of T7 phages under the influence of N-acetylaethylenimine have been determined.

A direct inactivation to be measured immediately after the treatment and an after-effect to be observed during incubation following the treatment have been distinguished.

On the basis of the curves obtained the quantities characterizing the phases of inactivation have been determined.

Study of the Radiosensitivity of Valyl-tRNA Synthetase

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Valyl-tRNA synthetase was isolated from chicken embryo brain by ultracentrifugation and fractionation on DEAE cellulose column. In the presence of 20 per cent glycerol and 6 mM mercapto-ethanol the enzyme can be stored at $-20°C$ without any loss of activity for several months. The effects of ^{60}Co-γ-radiation (NORATOM, 270 rad/sec dose rate) were studied in one part of the experiments only in the presence of phosphate buffer, in the other part, in the presence of glycerol and mercapto-ethanol.

The results obtained might be summarized as follows:

1. Upon the effect of ^{60}Co-γ-radiation in the range from 10.000 to 200.000 rads the biological activity of the enzyme decreases exponentially as a function of the dose.

2. The first step of enzyme activity was measured by the valine-dependent ATP-PP_i exchange reaction. The radiosensitivity of this enzyme function is appreciably lower than the radiosensitivity of the complete reaction: the formation of valyl-tRNA.

3. Both glycerol and mercapto-ethanol exert intensive radioprotective effects.

4. Also the enzyme concentration during irradiation essentially affects its inactivation rate.

5. According to the comparative study of enzymes isolated at various stages of embryonic development the activity of the enzyme isolated from the brains of younger (15 days old) embryos is by 20—25 per cent higher than the activity of enzymes from 19 days old embryos.

Comparison of our Radiation Kinetic Model with Radiochemical Results Obtained on RNA

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A model has been constructed to interpret the mechanism of the UV radiation injury of MS2 phages, and to investigate the relation between the radiation damages of structure and function. This model and a modified version of it were applied to compare the radiochemical results obtained in other laboratories (Cerutti et al.) with our own ones. The authors cited showed that the UV light of 280 nm only created hydrated photoproducts in R17 phage RNA as detected by chemical methods. On the basis of our model we have shown these experiences to be in good agreement with our results. Low doses of UV light causing well measurable phage inactivation form both irreversible (hydrated) photoproducts and photodimers revertable by UV light. According to our experiences both photoproducts cause phage inactivation.

A Molecular Enzyme Model Based on Oriented Energy Transfer

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A great number of theories exists to explain the rate acceleration action of enzymes.

Most of them comment this effect of enzymes as to be yielded by the specific circumstances of formation of the enzyme–substrate complexes (e.g.: orbital steering, proximity, strain, etc.).

The model presented considers the possibility and consequence of a specific energy transfer between the enzyme molecule and its environment (i.e., the molecules of the solution).

The energy of enzyme action comes from the transfer of translation energy of solute molecules into the vibration levels of the enzyme–substrate complex, namely the vibration levels of atoms oscillating along the intermolecular bonds (T—V transition). The specific structure of the globular proteins makes the existence of such an energy transfer mechanism very likely. The above phenomenon has an influence on the mean life-time of enzyme–substrate complexes as it follows from the mathematical description of the problem. Considering this kind of energy transfer a new type of enzyme regulation by the surrounding medium appears. It has been found that both preexponential and exponential factors of the equation describing the k_2 rate constant (the proportionality factor of the product formation) have parameters depending on the mass composition of the solution.

Effect of Viscosity on the Kinetic Parameters of Enzyme Reactions

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The viscosity of the physiological environment of enzymes is remarkably higher than that of the media in which they work in vitro. This is the reason why the number of publications dealing with the effect of viscosity on the kinetic parameters of enzymes is rapidly increasing.

The present paper reports some theoretical and experimental data on this problem.

The Michaelis—Menten type reactions as modified by Briggs and Haldane have been studied in a theoretical way: $E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + Pr.$

It was tried to clarify the apparent alteration of the kinetic constants caused by the increasing viscosity, i.e., the connection between the measured and intrinsic kinetic constants.

Results:

$$T' = t' \frac{k_{-1} + k_2}{pk_{-1} + k_2}$$

$$K_2 = k_2 \quad \text{and} \quad K_{-1} = pk_{-1}$$

where t' and T' are the real and measured average life time of the ES complex, K_2 and K_{-1} are the measured kinetic constants and p is a parameter dependent on temperature, viscosity and some other characteristic features of the system.

The hydrolysis of NPA, catalyzed by chymotrypsin has been investigated. The viscosity of the environment has been increased by adding PEG to the reaction mixture. The kinetic measurements have been carried out photometrically.

The data obtained by the measurements prove the decomposition rate of the complex to change as a function of the concentration of PEG. It is not a monotonous function, having an external value at certain concentrations of PEG. It might at first suggest that in case of the reaction mentioned at least two effects are of importance.

Book Reviews

Susan Hollán: Haemoglobins and Haemoglobinopathies. Publishing House of the Hungarian Academy of Sciences, 1972 (in Hungarian). 280 pages, 128 figures, 24 tables 547 references

Nowadays in spite of the fascinating development in biology and especially in molecular biology the application of the latest results in medical practice cannot yet be regarded as really widespread. This contradiction may be solved if among others, the activity of experts, accompanied by proper scientific propaganda, makes the clinicians conscious of the indispensability and paramount importance of the application of theoretical results. Susan Hollán's review on haemoglobins is useful and important work just because it favourably unites the two aims mentioned above the application of the results of theoretical and experimental research in clinical work. The volume convincingly illustrates the fact that the practitioner if he really intends to achieve good results, cannot ignore the newest findings in various areas of basic research, such as biochemistry, cytology and genetics. In fact we might perhaps say that this is the only way to change the many centuries old empirical approach to a strive for exactness that satisfies modern standards.

The beautiful presentation of the book bearing witness to the generosity of the editor, and the content reflecting the comprehensive and profound knowledge of the author will undoubtedly make this work an indispensable source for all those who in one way or another come across the problem of haemoglobins in the course of their work. Besides, the work raises novel problems that may determine the scientists' activity for the future.

In the light of current research the book expounds very clearly the role of haemoglobins, the oxygen transport proteins, their formation and evolution and describes the details of protein structure based on comparative protein analytical and X-ray diffraction studies. The first chapter already convinces the reader that the question, similarly to others, can only be grasped in its complex connections, and for that a broad spectrum of natural sciences has to be applied. Further, based on both the literature and own research work the author analyses the anomalous and pathological forms originating from the intraspecies polymorphism of haemoglobines. S. Hollán writes in detail about the probable mechanism of formation of pathological haemoglobins and about the ultrastructure of the erythrocytes containing such proteins, and also about the clinical aspects of haemoglobinopathies which is indispensable for the physician. Separate chapters deal with congenital cyanosis and polyglobulia. In the end she discusses the genetics of the development of populations containing anomalous haemoglobins and the circumstances of their geographical distribution which are all necessary aspects for the understanding of this problem complex. Although in Hungary anomalous haemoglobins are not as frequent as elsewhere, the acquaintance with the experiences gathered from them is very instructive. This is especially true, if we think of the fact that in the future we may diagnose further molecular diseases to the understanding of which the knowledge on haemoglobins may contribute a great deal.

An outstanding value of the book is the detailed and comprehensive laboratory and clinical analysis of own cases. The high quality illustrations, the extensive documen-

tation and the abundance of references will certainly render this book a useful and well applicable aid for all those interested in haemoglobinopathies and molecular diseases.

P. ELŐDI

Medizinische Physik. Einführung in die biophysikalische Analyse medizinischer Systeme. W. Beier, K. Döhnert, M. Rödenbeck. VEB Gustav Fischer Verlag, Jena. 1972; 531 pp., 267 figures and 40 tables

It has been the aim of the authors of the book of medical physics containing 531 pages, 267 figures and 40 tables to write a manual which suffices the higher requirements and modern forms of teaching needed by the reform of teaching at the universities everywhere nowadays; to summarize the necessary knowledge in physics and apply them for the human organism. On the other hand the authors chose the length of the book and detailed the treated problems so that the readers can use it as a guide in certain special problems during their later studies.

The structure of the book differs from the usual structure of books of experimental physics and medical physics in two respects. Human organism as a biophysical system is put in the centre, the book starts from the general characterization of the system, then treats its macroscopic and microscopic division always laying stress on physical analysis.

The book is divided into seven chapters:

1. The whole system — a description and analysis of medical systems according to general physics (M. Rödenbeck).
2. Physical description and analysis of the macroscopically divided systems of medical physics (M. Rödenbeck).
3. Microscopic sub-systems (W. Beier).
4. Vibrations and waves — manual for the physical analysis of function of medical systems (K. Döhnert).
5. Integrating sub-systems — Introduction into the biophysical analysis of function of medical systems (K. Döhnert).
6. Interaction between biological systems and physical forms of energy (W. Beier).
7. Introduction into medical data processing (K. Döhnert).

In the first chapter, after the definition of the system and the characteristics of the system the author expounds the elements of kinematics with the usual method of treating of experimental physics. This is followed by the treatment of the general characteristics of material systems such as geometrical characteristics, mass, weight, temperature, electric features, determinatives of state of gases and equation of state. The last part of the first chapter deals with the elements of bioenergetics, such as work, energy, thesis of energy, biocalorimetry; then it deals with the thesis of entropy in closed and open systems and with thermodynamical potentials and equilibriums. In this chapter the author has a sense for finding the balance between the communication of formal knowledge and the expounding of the basic idea chosen as the aim of the book.

The first part of the second chapter begins with the treatment of the interaction of complex systems (static and dynamic), this is followed by a chapter about the methods of determining the macroscopic structure, which deals chiefly with the optical projection, the characteristics and processing of photographic plates, and the modern methods of projection of clinical diagnostics. The third sub-chapter gives an inside view of the material characteristics and their determination (notions of concentration, optical material constants, such as refractivity, absorption coefficient, specific optical activity, viscosity, electric and thermodynamic characteristics of material. The second chapter is finished with the treatment of some problems of biomechanics and model-forming.

The authors — according to the aim of the chapter (expounding of regularities and characteristic features corresponding to the macroscopic distribution of the biological system and of methodical problems connected with this) — undertook a difficult task within the given frame. Certain sub-chapters offer a closed unit for the reader, at the same time other sub-chapters as e.g. chapter 2.3.2, the physical bases of which can be found at another place of the book, are rather restricted.

The third chapter is divided into five sub-chapters. The first one deals with the interactions going on in microscopic subsystems; diffusion and osmosis, active transport, Donnan equilibrium, membrane potential

and mechanochemical processes, first of all the activity of muscle. The second sub-chapter practically summarizes light microscopic physical knowledge, including phase-contrast, polarization, and interference microscopy. There is a short summary about X-ray and electron microscopy. The third sub-chapter deals with the physical features of biomolecules: structure of proteins and their optical characteristics, structure of nucleic acids and their reduplication. The fourth one gives an inside view of the physical characteristics of molecular and atomic systems; it treats radioactivity and the elements of nuclear physics. The chapter closes with a short passage on elementary particles. It must be mentioned in connection with the chapter that it does not treat, or treats only briefly, the knowledge of molecular physics which would be inevitably necessary for the establishment of modern molecular biophysics.

The most homogeneous and clearly arranged part of the book is the fourth chapter constituting about a quarter of the book and giving a detailed treatment of the analysis of periodic processes taking place in the organism and connected with human organisms such as the physical bases of the activity of heart and blood circulation, hearing and speaking. It discusses very thoroughly the characteristic features of recording systems and their structure, certain coders, the amplifying equipments necessary for processing of signals. The author devotes a separate chapter to electromagnetic waves and within this frame he deals with the elements of physical optics and gives an inside view of holography.

In chapter five we find an introduction into the elements of information theory and regulation circles, the treatment of which is supplemented by biological examples. The author devotes a separate sub-chapter to the physical references of hearing.

The sixth chapter practically discusses three problems under the title: interaction of biological systems and physical forms of energy. It expounds X-ray radiation, the elements of dosimetry and primary radiation effect; it deals with high-frequency therapy and gives a survey of ultra-sound therapy.

The seventh chapter treats an important problem; it presents the possibilities and concrete forms of application of data processing by computers and computer diagnostics. It expounds the basis of both the digital and analogous information processing. The expounding is completed by some examples.

At the end of the book we find a detailed subject index. But it is regrettable that the authors do not serve with a list of references at the end of the chapters or the book which would have the purpose of helping the readers in finding appropriate books of reference for obtaining new knowledge or finding additional material to the shortly written passages. We recommend the book to medical students, attention for their obtaining the necessary bases of physics and medical technique beside their biophysical knowledge.

J. BELÁGYI

Grundlagen der Genetik und Züchtung industriell genutzter Mikroorganismen. S. I. Alichanjan. VEB Gustav Fischer Verlag, Jena, 1972, 351 pp., 111 figures and 132 tables.

The book consisting of 351 pages, printed on fine paper with an excellent typography, and containing 111 figures and 132 tables is the translation of a Soviet publication appeared in 1968.

The work evidently aims at providing industrial experts having interest, or being interested, in the basic problems of genetics connected with microbiological products with an easily understandable guidance. The work comprises three large chapters (2, 3, 4); fundamental conceptions of genetics (i), utilization of methods of genetics in procedures of culturing (ii), special problems: microbial synthesis of antibiotics, vitamins, enzymes, etc. (iii). These chapters are completed with lists of references, microbe-species and subjects.

The first (2) chapter treats fundamental conceptions of genetics. The historical survey sometimes seems somewhat oversized; instead of this it would seem more important to expound and illustrate notions as e.g. frequency of mutation (and not the frequency referred to the number of colonies). A certainly great merit of this chapter is the short and perfect expounding of the genetics of Actinomycetae, which can be found in few handbooks only.

The next (3) chapter deals with the selection and induction of economically useful mutants — mainly on an empirical basis. Again, the historical survey seems to be too much: e.g. during the expounding the lethal and mutagenous effect of ultraviolet irradiation the author mentions thymin-dimers but after having written 17 pages. On the other hand he just mentions the photoreactivation- induced enzyme-splitting, the "dark" reparation excision is only hinted at, while the reparation of recombinatory mechanism is not even mentioned, even though that is the source of origin of the overwhelming majority of mutants. The statement (3.9) that strains become phage-resistant (immune in the proper sense) under the effect of lysogenization is misleading; under resistance we mean today a change resting upon the receptor, as it becomes evident from the further discussions of the author.

In the last (4) chapter we find a description of different strains producing industrially valuable products, and of the production of mutants offering multiple of natural synthesis. In this theme of industrial significance also the historical survey has naturally a significant role, but the author also gives an excellent scheme of performing this kind of work. This chapter connected with the practice seems to be the most intimate for the author and, accordingly it is the most successful chapter of the book.

It is somewhat unusual to separate Soviet and not Soviet literature in the list of references. This can be useful for home readers, but it would be better to avoid it in translation. Incidentally, the titles of communications in Russian had been translated into German.

I. KÉTYI

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MATHEMATICAL MODELS OF METABOLIC REGULATION

Post-Congress FEBS Advanced Course No. 27

will be held in Hungary from 1 to 5 September, 1974.

The 9th FEBS Meeting (Budapest, August 25 – 30, 1974) will be correlated with the Advanced Course. Part of the Course will constitute of seminars on lectures given in Symposium 2 (Mechanism of Action and Regulation of Enzymes) and Colloquium III (Analysis and Simulation of Biochemical Systems) of the Meeting. Students are recommended to attend these lectures. Second part of the Course will include lectures on selected chapters of enzyme kinetics and analysis of metabolic systems not discussed at the Meeting.

The fee for scholars will be 170 dollars or equivalent, which includes the registration fee for the 9th FEBS Meeting and accommodation and meals during the Advanced Course (but *not* during the Meeting!). The papers presented at the FEBS Advanced Course No. 24 on Mathematical Models of Metabolic Regulation (Oberhof, GDR, 1972) are available at a modest price from Prof. Dr. H. Frunder (Physiol.-Chem. Inst. der Friedrich Schiller Univ. 69 Jena. Zentraler Platz. GDR).

The number of students is limited. Correspondence should be sent as soon as possible to FEBS Advanced Course, c/o Dr. T. Keleti, Enzymol. Dept., Inst. Biochem. Hung. Acad. Sci. H-1502 Budapest, Pf. 7. Hungary.

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The Tryptic Digestion of Phosphorylase-*a* and *b*

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Institute of Medical Chemistry, University Medice School Debrecen, Hungary.

(Received November 19, 1972)

The limited tryptic proteolysis of phosphorylase-*a* and *b* was studied by measuring the enzymatic activity of the *a*-form and the phosphorylation of the *b*-form.

The results confirm that tetrameric phosphorylase-*a* is resistant to tryptic attack. The tryptic digestion of dimer *a*, i.e. the formation of *b'*, is inhibited by AMP; the inhibition is not due to the formation of the tetrameric species but to the conformational change of dimer *a*. Glucose, glucose-6-phosphate and caffeine increase the digestion of dimer *a* and suspend the inhibitory effect of AMP.

The phosphorylase-*b* → *b'* conversion is not, or is only slightly inhibited by ATP and AMP, and is not promoted by glucose and glucose-6-phosphate. The activity of phosphorylase-*b'* whether derived from phosphorylase-*a* or from phosphorylase-*b*, could be inhibited allosterically by ATP and glucose-6-phosphate. On this basis the two phosphorylase-*b'*-s produced in different ways seem to be identical.

The effectors that do not affect the phosphorylase-*b* → *b'* conversion induced such a conformational change in the *b'*-form which protects this species from further tryptic degradation. The observation made by several authors that the proteolysis of phosphorylase-*b* could be inhibited by effectors, can in fact be attributed to the inhibition of further digestion of phosphorylase-*b'*.

Introduction

It has been shown that the active, phosphorylated *a*-form of phosphorylase is converted to phosphorylase-*b'* by trypsin, which form is only active in the presence of AMP (Cori, Cori, 1945; Keller, 1955), similarly to phosphorylase-*b*. During the course of *a* → *b'* conversion by trypsin one hexapeptide per subunit is removed, which contains the phosphorylated serine residue and whose amino acid sequence has already been determined (Fischer et al., 1959). Thus the amino acid sequence is known around the residue which is phosphorylated in phosphorylase-*b* by phosphorylase-*b* kinase, and is dephosphorylated in phosphorylase-*a* by phosphorylase phosphatase.

It has been demonstrated that glucose increases and AMP inhibits the phosphorylase-*a* → *b'* conversion. From this it was concluded that the modification of tryptic digestion was due to conformational alterations of phosphorylase-*a*, i.e. phosphorylase-*a* exists in different conformational states in the presence of glucose, or AMP, or glucose + AMP (Graves et al., 1968; Wang, Black, 1968; De Barsy et al., 1972).

Phosphorylase-*b'* resembles in many respects phosphorylase-*b*, which is formed from phosphorylase-*a* by dephosphorylation: their molecular weights and enzymatic activities in the presence of AMP are identical. However, phosphorylase-*b'* cannot be converted back to phosphorylase-*a* by phosphorylase-*b* kinase, because the hexapeptide containing the serine residue is missing from it. According to Graves et al. (1968) phosphorylase-*b'* also differs from phosphorylase-*b* in that it has no allosteric properties. The *b'*-form derived from *a* could not be inhibited by glucose-6-phosphate. Graves et al. (1968) inferred that the hexapeptide around the phosphorylatable serine "is an essential part of the molecular structure which is responsible for the regulatory behavior of glycogen phosphorylase".

The tryptic digestion of phosphorylase-*b* has been much less studied than that of the *a*-form presumably because the proteolysis of phosphorylase-*b* is more difficult to follow: the *b'*-form produced has the same activity as the initially present phosphorylase-*b*.

First spermine was used to follow the effect of trypsin since this substance can only enhance the activity of phosphorylase-*b* but not of *b'* (Wang et al., 1968). By this method it has been shown that the *b'* derived from *b* cannot be transformed to the *a*-form.

It was demonstrated that certain effectors (AMP, ADP, glucose-6-phosphate and glucose) inhibit the tryptic digestion of phosphorylase-*b* (Muszbek et al., 1968; Muszbek, Sümegi, 1970; Kurganov et al., 1972). In these studies, however, the influence of effectors on the first step of digestion, on the $b \rightarrow b'$ conversion, could not be separately detected. Namely, proteolysis was followed by determining the amount of TCA-precipitable protein and of solubilized peptides, or by measuring the decrease of fluorescence of phosphorylase-*b* and therefore the results must have been influenced more heavily by the further digestion of *b'*-form than by the $b \rightarrow b'$ conversion itself.

In the present work we studied the effect of the various effectors on the $b \rightarrow b'$ conversion, comparing it with the $a \rightarrow b'$ conversion. Our aim was also to examine the allosteric behaviour of *b'* derived from *b*, and to compare it with that of *b'* obtained from *a*.

Materials and methods

Phosphorylase-*b* kinase was prepared from rabbit muscle and the purified enzyme was activated in the presence of Ca^{2+} according to Krebs et al. (1964). The specific activity of the preparation was 89 units per mg of protein. For one activity unit of phosphorylase-*b* kinase was taken the amount of enzyme that converted 1 unit of phosphorylase-*b* to *a* in 1 minute.

Phosphorylase-*b* was also prepared from rabbit muscle by a modification (Bot et al., 1971) of the method of Fischer and Krebs (1962). The specific activity of the three times recrystallized preparation was 44 units per mg protein. One unit of phosphorylase-*b* liberates 1 μmole of P_i in 1 minute in the presence of AMP under the conditions of the activity assay.

Phosphorylase-*a* was obtained from phosphorylase-*b* prepared by us by treatment with phosphorylase-*b* kinase as described by Krebs and Fischer (1962). The crystalline product had a specific activity of 55 units per mg of protein. One unit of phosphorylase-*a* liberates 1 μmole of P_i in 1 minute in the absence of AMP.

The *dimeric form* of phosphorylase-*a* was produced by incubating phosphorylase-*a* at 36 °C for 15 minutes after dilution in a buffer (0.04 M Tris, 0.01 M mercaptoethanol, 0.002 M EDTA, pH 6.8) containing 1% glycogen. The presence of glycogen ensures the maintenance of dimeric state after the incubation period when the mixture is cooled to 18 °C. *Tetrameric* phosphorylase-*a* was obtained by dissolving the preparation in glycogen-free buffer at 36 °C and then cooling the solution to 18 °C. Only then was 1% glycogen added to the solution which did not cause the dissociation of tetramer, i.e. phosphorylase-*a* at 18 °C.

Assay of phosphorylase activity

The activity of phosphorylase-*a* and *b* was measured in the direction of glycogen synthesis by the method of Illingworth and Cori (1953). The assay mixture contained 16 mM glucose-1-phosphate, 1% glycogen and an amount of phosphorylase to convert not more than 20% of glucose-1-phosphate during the incubation period. The activity of phosphorylase-*a* was measured without AMP, that of phosphorylase-*b* in the presence of 1 mM AMP.

Inorganic phosphate (P_i) was determined according to Taussky and Shorr (1953).

Protein concentration was measured spectrophotometrically at 280 nm, by using the extinction coefficient $E_{280}^{1\%} = 11.7$ (Velick, Wicks, 1951). The specific activities of enzymes were calculated from the protein contents thus determined.

Tryptic digestion of phosphorylase-a and b

Phosphorylase-*a* was digested with trypsin at 18° and 30 °C, phosphorylase-*b* at 30 °C. The digestion mixture contained in general 20 units/ml dimeric phosphorylase-*a* or 100 units/ml phosphorylase-*b*, and 10 μg/ml of trypsin in 0.04 M Tris, 0.01 M mercaptoethanol, 0.002 M EDTA buffer, pH 6.8. The effectors were dissolved in the above buffer and added to the digestion mixture, to a final volume of 0.8 ml.

At suitable time intervals aliquots were withdrawn and diluted in a solution of soybean trypsin inhibitor dissolved in the above buffer, in order to arrest trypsin action (trypsin/trypsin inhibitor ratio = 1 : 5, w/w). Digestion was followed by measuring the residual phosphorylase-*a* activity, or by measuring the convertibility of residual phosphorylase-*b* to *a*.

Determination of phosphorylase-b by conversion to a

During the incubation of phosphorylase-*b* with trypsin aliquots were withdrawn from the mixture to assay the amount of undigested phosphorylase-*b*.

To the aliquot (maximally 80 units phosphorylase-*b*) trypsin inhibitor (trypsin/trypsin inhibitor ratio = 1 : 5), then 3 mM ATP and 10 mM Mg^{2+} were added. Finally phosphorylase-*b* kinase was added in excess (about 15 units) so as to completely convert undigested phosphorylase-*b* to *a* at 30 °C in 10 minutes. The phosphorylase-*a* produced was determined by enzymatic assay and this served as a measure of undigested phosphorylase-*b*.

Preparation of phosphorylase-b'

Twice recrystallized phosphorylase *b* (500 units), or phosphorylase-*a*, was incubated with 40 μg of trypsin in 0.04 M Tris buffer, pH 6.8, containing 20 mM glucose at 30 °C for 10 minutes, in a final volume of 1 ml. At the end of incubation the tryptic effect was stopped by the addition of soybean trypsin inhibitor in excess. The mixture containing phosphorylase-*b'* was chromatographed on a Sephadex G-100 column in 0.04 M glycerophosphate, 0.01 M mercaptoethanol, 0.002 M EDTA buffer, pH 6.8. The phosphorylase-*b'* content of fractions (2 ml each) was determined on the basis of enzyme activity with AMP, and it was checked that the product could not be re-converted to phosphorylase-*a* by phosphorylase-*b* kinase. In this way we obtained a phosphorylase-*b'* preparation of specific activity 37 units per mg of protein.

Results and discussion

Effect of dimeric and tetrameric states on the digestion of phosphorylase-a

In accordance with the fact that glucose enhances the tryptic digestion of phosphorylase-*a*, which can partly be attributed to the dissociation of tetrameric *a* to dimers (Graves et al., 1968; Wang, Black, 1968; De Barsy et al., 1972), we could show that trypsin, even without effectors, could primarily digest dimeric *a*, whereas it hardly affected tetrameric *a* (Fig. 1).

It can be seen in Fig. 1 that the dimeric form of phosphorylase-*a* loses activity much faster than the tetrameric species. It can be assumed that the tetrameter is even less digestible than it seems, as phosphorylase still contains 20% dimers at 18 °C. The tetrameric form may dissociate during digestion owing to the disturbance of the equilibrium, but this is a slow process at 18 °C and may limit the rate of digestion of the tetramer.

Glycogen added to the tetramer later does not influence the effect of trypsin, since tetrameric *a* fails to form a complex with glycogen and thus glycogen cannot induce a conformational change.

Effect of glucose-6-phosphate and caffeine on the digestion of phosphorylase-a

As mentioned in the Introduction the influence of certain effectors such as glucose and AMP in the tryptic digestibility of phosphorylase-*a* has already been studied. However, the effect of glucose-6-phosphate and caffeine has not

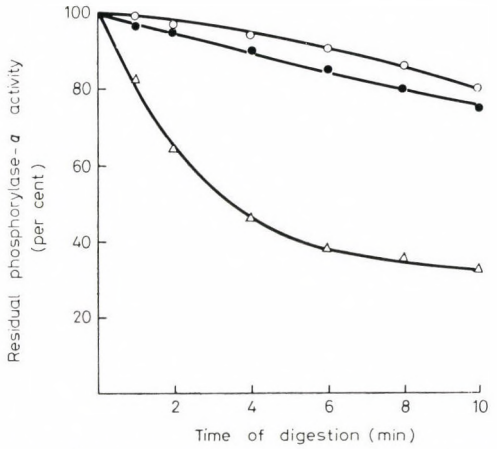

Fig. 1. Tryptic digestion of dimeric and tetrameric phosphorylase-*a* at 18 °C. Digestion mixture: 100 units/ml of phosphorylase-*a* (specific activity 55 units/mg); 20 μg/ml of trypsin in 0.04 M Tris, 0.01 M mercaptoethanol, 0.002 M EDTA buffer, pH 6.8. Digestion was stopped by the addition of trypsin inhibitor. The samples were diluted according to the residual phosphorylase-*a* activity, which was assayed by the method of Illingworth and Cori (1953). The dimeric and tetrameric forms of phosphorlyase-*a* were prepared as described in Methods. △—△ dimer *a*; ○—○ tetramer *a*; ●—● tetramer *a* + 1% glycogen

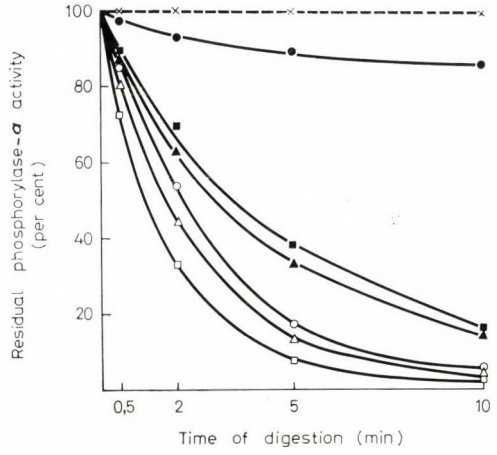

Fig. 2. Effect of caffeine and glucose-6-phosphate on the tryptic digestion of phosphorylase-*a*. Digestion mixture: 20 units/ml phosphorylase-*a*, 10 μg/ml of trypsin in 0.04 M Tris, 0.01 M mercaptoethanol, 0.002 M EDTA buffer, pH 6.8. The effectors were dissolved in the above buffer and then added to the mixture. Incubation was carried out at 30 °C. At the times indicated aliquots were withdrawn; digestion was stopped and the residual phosphorylase-*a* activity was assayed as described in the legend to Fig. 1. ○—○ digestion without effector; □—□ digestion with 5 mM caffeine; △—△ 15 mM glucose-6-phosphate; ●—● 10^{-5} M AMP; ■—■ 10^{-5} M AMP + 5 mM caffeine; ▲—▲ 10^{-5} M AMP + 15 mM glucose-6-phosphate, activity assay in the above cases without AMP; ×- - × digestion in the presence or absence of effectors, activity assay with 1 mM AMP

yet been examined. It is known about caffeine that it inhibits the activity of phosphorylase-*a* (Kihlman, Overgaard-Hansen, 1955; Alpers et al., 1963) and promotes the inactivation of phosphorylase-*a* by phosphatase (Stalmans et al., 1970), but no data have been provided on whether it influences tryptic digestibility, and whether it induces conformational alterations in the phosphorylase-*a* molecule.

The effect of glucose-6-phosphate has not been investigated presumably because it does not considerably affect the activity of phosphorylase-*a* (Morgan, Parmeggiani, 1964) although recently it has been shown that it is able to inhibit the activity of the partially phosphorylated form (Hurd et al., 1966; Livanova et al., 1972). Thus it is particularly important whether it influences tryptic digestion, which is a sensitive indicator of conformational changes.

Fig. 2 shows the effect of caffeine and glucose-6-phosphate on the tryptic digestion of rabbit muscle phosphorylase-*a*.

It is seen that caffeine and glucose-6-phosphate increase the proteolysis of phosphorylase-*a* by trypsin. Both effectors greatly decrease the inhibitory effect of AMP on digestion.

Since the effectors influence already the first step of digestion, the $a \rightarrow b'$ conversion, their effect can be ascribed to some conformational change in phosphorylase-*a*. This is supported by our observation that only the so-called *a*-activity, without AMP, decreases during digestion, while the activity that can be attributed to phosphorylase-*b'* measured in the presence of 1 mM AMP does not change.

On this basis it can be assumed that the conformational change induced by AMP results in the "burying" of the hexapeptide that is cleaved off by trypsin. On the other hand, caffeine and glucose-6-phosphate elicit such conformational alterations that render the hexapeptide more readily accessible to trypsin.

The accelerating effect of glucose-6-phosphate on digestion is only little, but it markedly affects the inhibitory effect of AMP on digestion, almost cancels it. This observation suggests that glucose-6-phosphate is capable of changing the conformation of phosphorylase-*a* assumed in the presence of AMP.

However, glucose-6-phosphate is not a suitable effector of all kinds of phosphorylases. For example, unlike with rabbit muscle phosphorylase, it fails to influence the activity of pig muscle phosphorylase. Accordingly, it does not affect the tryptic digestion of pig muscle phosphorylase-*a* and does not suspend the inhibitory effect of AMP on digestion either (Varsányi, Bot, 1971).

Tryptic digestion of phosphorylase-b

It is in these experiments the first time that the tryptic digestion of phosphorylase-*b* was followed by measuring the decrease in the amount of phosphorylase-*b* via conversion to *a*. Aliquots were taken from the phosphorylase-*b* sample incubated with trypsin and the undigested phosphorylase-*b* was converted to phosphorylase-*a* by the aid of phosphorylase-*b* kinase. From the activity of phosphorylase-*a* produced did we infer as to the amount of undigested phosphorylase-*b* (Fig. 3).

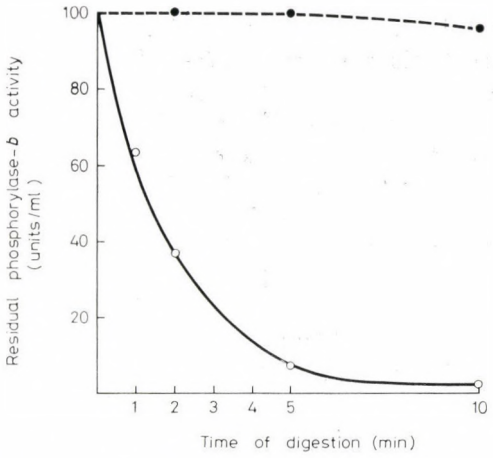

Fig. 3. Measurement of the tryptic digestion of phosphorylase-*b* by the kinase reaction. Digestion mixture: 100 units/ml of phosphorylase-*b* (specific activity, 44 units/mg); 10 μg/ml of trypsin in 0.04 M Tris, 0.01 M mercaptoethanol, 0.002 M EDTA buffer, pH 6.8. Incubation was carried out at 30 °C. Digestion was stopped by the addition of trypsin inhibitor and residual phosphorylase-*b* was converted to phosphorylase-*a* by phosphorylase-*b* kinase (cf. Methods). Phosphorylase-*a* produced was assayed as described in Fig. 1. ○—○ residual phosphorylase-*b*; ●—● activity measured in the presence of 1 mM AMP (residual *b* + produced *b'*)

It can be seen in Fig. 3 that on the effect of trypsin the amount of residual phosphorylase-*b* decreases, as it loses its ability to be converted to *a*. The enzymatic activity measured in the presence of AMP remains unchanged, that is phosphorylase-*b'* is produced, i.e. the $b \rightarrow b'$ conversion took place.

Measuring the $b \rightarrow b'$ conversion by the above method we tested the influence of certain effectors (Table 1).

Table 1

Influence of effectors on the rate of $b \rightarrow b'$ conversion

Digestion mixture: 100 units/ml phosphorylase-*b*; 10 μg/ml trypsin, in 0.04 M Tris, 0.01 M mercaptoethanol, 0.002 M EDTA buffer, pH 6.8. Incubation was carried out at 30 °C. The effectors were dissolved in the above buffer and were added to digestion mixture. At times aliquots were withdrawn to determine the activity of undigested phosphorylase-*b*, as described in Fig. 3. The data thus obtained were used for the calculation of the first order rate constants of digestion

Effector	Rate constant (min^{-1})
Without effector	0.50
Glucose-1-phosphate, 20 mM	0.48
Glucose, 20 mM	0.49
Glucose-6-phosphate, 10 mM	0.55
ATP, 10 mM	0.58
AMP 1 mM	0.28

As shown in Table 1, the effectors used did not markedly affect, even at relatively high concentrations, the rate of $b \rightarrow b'$ conversion. AMP, which protects phosphorylase-*a* from digestion at a concentration as low as 10^{-5} M, barely somewhat decreases the rate of $b \rightarrow b'$ conversion even at 1 mM concentration.

On the basis of the foregoing the effectors that inhibit the digestion of phosphorylase-*b* according to certain authors (Muszbek et al., 1968; Muszbek, Sümegi, 1970; Kurganov et al., 1972) inhibit in fact not the $b \rightarrow b'$ conversion proper, but rather the further digestion of the *b'* form. Consequently, their effect resides not in changing the conformation of phosphorylase-*b*, but rather that of phosphorylase-*b'*.

Phosphorylase-*b'* produced by the tryptic digestion of phosphorylase-*b* can thus be characterized by stating that it displays enzyme activity in the presence of AMP, but cannot be reconverted to phosphorylase-*a*. It appears that in its basic properties it is identical with *b'* derived from phosphorylase-*a*. The question arose whether the two phosphorylase-*b'*-s, obtained from *a* and *b*, had also the same allosteric features or not.

The allosteric properties of phosphorylase-b'

Phosphorylase-*b'* preparations were produced from phosphorylase-*a* and *b* with trypsin (cf. Methods) and their properties were compared (Fig. 4).

Fig. 4 shows the substrate saturation curves of phosphorylase-*b'* preparations obtained from phosphorylase-*b* (A) and phosphorylase-*a* (B). The curves become sigmoidal in the presence of effectors. ATP inhibits pronouncedly the activity of both *b'* preparations although it is known as an allosteric inhibitor

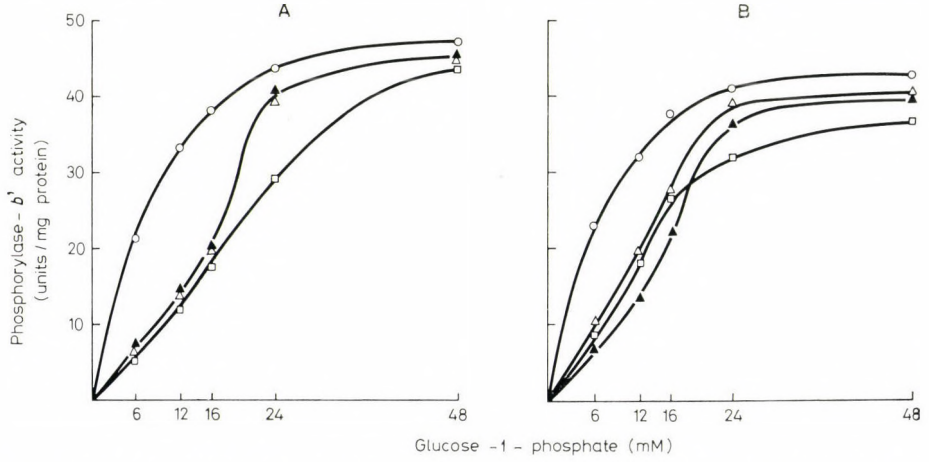

Fig. 4. Allosteric properties of *b'* preparations obtained from phosphorylase-*b* (A) and phosphorylase-*a* (B). The two phosphorylase-*b'* preparations (A and B) were produced as described in Methods. Their activities were measured in 0.04 M Tris, 0.01 M mercaptoethanol, 0.002 M EDTA buffer, pH 6.8, with increasing concentrations of glucose-1-phosphate in the presence of 1 mM AMP, with or without effectors. Activity: ○—○ without effectors; △—△ with 10 mM glucose-6-phosphate; □—□ 20 mM glucose; ▲—▲ 10 mM ATP

of only phosphorylase-*b*. Glucose slightly inhibits and in its presence the cooperative interaction is less conspicuous. The effect of glucose-6-phosphate is the most interesting, inasmuch as it also inhibits the activity of *b'* derived from phosphorylase-*a*. It can be concluded that in this respect the *b'*-s do *not differ* from each other.

Our results are at variance with the data of Graves et al. (1968), as the *b'* prepared from phosphorylase-*a* by the named authors failed to display cooperative interactions and could not be inhibited by glucose-6-phosphate.

Tryptic digestion of phosphorylase-b'

On further treatment with trypsin phosphorylase-*b'* loses its enzymatic activity measured in the presence of AMP. We observed that to achieve this considerably higher trypsin concentration was needed than to the $b \rightarrow b'$ conversion. As mentioned above the effectors of phosphorylase-*b* did not influence the $b \rightarrow b'$ conversion, therefore we examined whether these effectors had any influence on the tryptic digestion of phosphorylase-*b'* (Fig. 5). It is seen that ATP, AMP and glucose-6-phosphate completely, whereas glucose and glucose-1-phosphate greatly protect phosphorylase-*b'* from tryptic attack.

The results explain those data in the literature according to which the tryptic degradation of phosphorylase-*b* could (apparently) be inhibited by the effectors. The protective effect of these compounds is in fact exerted not at the $b \rightarrow b'$ conversion, but only at the further digestion of phosphorylase-*b'*. Accord-

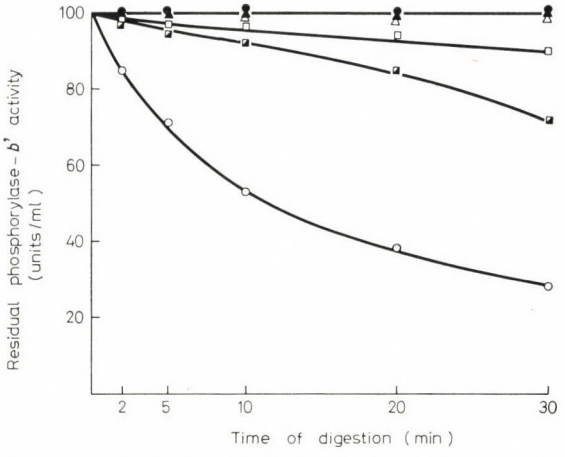

Fig. 5. Influence of effectors on the tryptic digestion of phosphorylase-*b'*. Digestion mixture 100 units/ml phosphorylase-*b'* (specific activity 37 units/mg); 60 μg/ml of trypsin, in 0.04 M Tris, 0.01 M mercaptoethanol, 0.002 M EDTA buffer, pH 6.8. Incubation was carried out at 30 °C. The withdrawn aliquots were diluted with a solution of trypsin inhibitor and the residual phosphorylase-*b'* activity was measured in the presence of 1 mM AMP. The effectors were dissolved in the above buffer. ○—○ digestion without effectors; △—△ digestion with 10 mM glucose-6-phosphate; □—□ 20 mM glucose; ■—■ 20 mM glucose-1-phosphate; ●—● 0.1 mM AMP; ▲—▲ 10 mM ATP

ingly, the tryptic removal of the hexapeptide, which accompanies the $b \rightarrow b'$ conversion, cannot be influenced by the effectors.

As phosphorylase-*b'* displays the same cooperative interactions as phosphorylase-*b*, the splitting off of the hexapeptide does not alter the allosteric properties of the enzyme molecule, i.e. the region containing the hexapeptide is not in close connection with those parts of the molecule that are responsible for the allosteric effects and for the enzymatic activity in the presence of AMP. The allosteric behaviour is only influenced by this region if the serine residue it contains is phosphorylated. Then the molecule becomes active even without AMP, and the allosteric effect by certain effectors is drastically reduced. Phosphorylation somewhat diminishes the exposed position of the hexapeptide, henceforth some effectors may promote, while others may interfere with the rate of $a \rightarrow b'$ conversion.

If the phosphorylated hexapeptide is removed, the molecule recovers its allosteric sensitivity, the cooperative interaction between the corresponding regions is restored. As a result, the properties of *b'* derived from phosphorylase-*a* are identical with those of *b'* obtained from phosphorylase-*b*; the *b'* preparations produced in the two different ways do not differ from each other.

Parallel with the phosphorylation of the hexapeptide the tendency of the phosphorylase molecule to associate also increases. As a result of association into tetramer the hexapeptide becomes "buried" and cannot be split off by trypsin any longer. That the hexapeptide may play a role in the process of association is also supported by the fact that phosphorylase-*b'* does not form tetramers, i.e. the removal of the hexapeptide is accompanied by the loss of associating ability.

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The Heterogeneity of Informofer

IV. Informofer Complexes Containing DNA

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Purified 30 *S* particles contain a small amount of DNA that shows a characteristic distribution pattern on gel-filtration on porous dextran or agarose gels. Digestion of the particles with DNAse of high purity eliminates only a part of the whole DNA and no change in the elution pattern of the particles is detectable.

If the DNAse treatment was accompanied by digestion with RNAse I, part of the particles was destroyed and the RNA that was resistant to RNAse I alone, was also hydrolyzed.

Introduction

The RNP-particles of the cell nucleus which carry the informational RNA also contain a small amount of DNA (Samarina et al., 1967; Naora, Kodaira, 1968). The role and properties of this DNA are not known yet. Moreover, the fact that the concentration of DNA is not constant either in the 30 *S* particles or in their polymers led to the assumption that here DNA is merely a contaminant.

Our present experiments seem to suggest that the DNA detected in the informofer complexes is not simply a contamination but may have some structural role. We found that part of informofer-bound RNA which was resistant to pancreatic ribonuclease was mainly hydrolyzed if DNAse was also present. On this basis it can be assumed that part of the RNA content of the informofer complex is present in that form of DNA–RNA hybrids, and this is of functional significance.

Materials and methods

Rats of both sexes weighing 150 to 180 g from the R Amsterdam strain were used. The RNP-particles were prepared from purified rat liver cell nuclei by the gel-filtration method described previously (Molnár, Komáromy, 1972). Aliquots (3 ml) of the purified particle suspension were analyzed by gel-filtration on a Bio-Gel A-5m column. The volume of the column was 150 ml, elution rate 16 ml/hour, eluting buffer: 0.01 M triethanolamine, 0.10 M NaCl, 0.001 M $MgCl_2$, pH 7.5. From the effluent 20-drop fractions (0.83 ml) were collected, and their absorbance at 230 and 260 nm, as well as their DNA content according to Ceriotti (1952) was determined. For the latter determination a calf thymus DNA standard was used. Radioactive labelling was performed either with (^{3}H)-thymidine (28.01

mCi/mg/ UVVVS, Prague) or in the case of double labelling of RNA with carrier-free (^{32}P)-orthophosphate and (^{14}C)-sodium orotate, as described previously (Molnár, Juhász, 1972). The radioactivity of samples was measured by a liquid scintillation counter (Gamma, Budapest), from the Synpor 2 cellulose nitrate membrane filtrate of the TCA-precipitated samples, in an apolar scintillation mixture (4 g of PPO, 100 mg of POPOP in 1000 ml of toluene).

In the experiments where DNAse was used alone a Worthington preparation of nominal purity was applied. Since the enzyme had no effect on the overall chromatographic pattern, it seemed to be indifferent whether it contained RNAse or not. For this reason in such cases the possible RNAse contamination of DNAse was not checked.

For digestion with DNAse in the presence of RNAse a Calbiochem B-grade enzyme preparation was used. Pancreatic RNAse and all the other chemicals (reagent grade) were purchased from Reanal (Budapest).

All operations during the isolation and chromatographic analysis of particles were performed between 0 and 4 °C.

Results

If the RNP-particles were chromatographed on a long, thin Sephadex G-200 column (1.2 × 140 cm), the frontal peak region was heterogeneous with regard to DNA content. Fig. 1a shows such a part of the elution pattern; the R_f difference between the 9th and 18th fractions is about 0.17. The main bulk of DNA is found in the foremost part of the peak. If the cell nuclei are extracted with 0.3 M NaCl (Molnár, Juhász, 1972) less DNA, and of lower molecular weight, is found in the chromatogram. This is all the more conspicuous as at such an ionic strength the probability of dissociation of nuclear DNP, and of the appearance of the dissociated DNA in the chromatogram, is greater. The elution pattern of particles extracted with 0.3 M NaCl is shown by Fig. 1b.

When the informofer particles purified on Sephadex were subjected to gel-filtration on a Bio-Gel A-5m column, the pattern illustrated in Fig. 2a was obtained. DNA content as determined according to Ceriotti shows two maxima. However, the radioactivity derived from labelling *in vivo* with 100 μCi of (^{3}H)-thymidine for 12 hours was only found in the second DNA peak. After treating the particles with DNAse (5 μg/ml for 15 min at room temperature) the elution pattern only changed in that the first DNA-peak was markedly diminished. There is no change in the ultraviolet absorption, and the distribution of radioactivity derived from thymidine incorporation is essentially also unaltered (Fig. 2b).

The chromatographic picture of doubly labelled particles is seen in Fig. 3. Naturally, here the incorporation goes mainly to RNA.

After treatment with RNAse (20 μg/ml, 20 min at room temperature) the ultraviolet picture of the chromatogram is not considerably changed, but the amount of labelled RNA is drastically reduced. If during hydrolysis DNAse is also present (20 μg/ml RNAse and 25 μg/ml DNAse, 20 min at room temperature) the pattern shown in Fig. 3c is obtained. Radioactivity derived from the

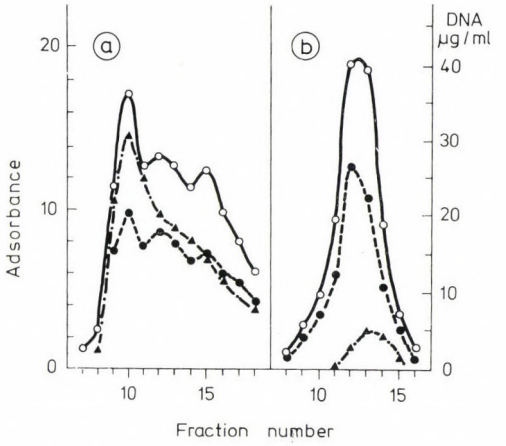

Fig. 1. Rechromatography on a Sephadex G-200 column (1.2×140 cm) of 0.1 (*a*) and 0.3 (*b*) nuclear extracts previously purified on a Sephadex G-200 column. Eluting buffer: 10^{-2} M triethanolamine, 0.1 M NaCl, 10^{-3} $MgCl_2$. ○—○ A_{230}; ●—● A_{260}; ▲ — · — · — ▲ DNA concentration, μg/ml

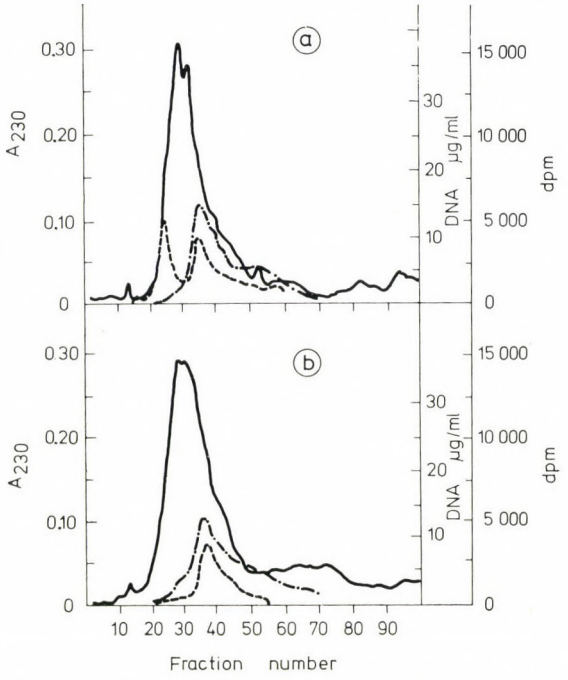

Fig. 2. Elution profile on a Bio-Gel A-5m column of particles purified by gel-filtration on Sephadex. Column size: 2.2×40 cm. —— A_{230}; — — — DNA concentration, μg/ml; — · — · — (^{3}H)-thymidine incorporation, dpm. *a*, control; *b*, DNA-treated sample, 5 μg/ml, 15 min at room temperature

two-hour and 20-min labelling further decreases, which indicates that DNAse rendered the residual part of RNA susceptible to the RNAse attack. The remaining (^{32}P) activity was probably incorporated not only into polyadenylic acid (Niessing, Sekeris, 1972) but also into the phosphates of nuclear proteins. It is interesting to compare the latter type of experiments under rigorously standard

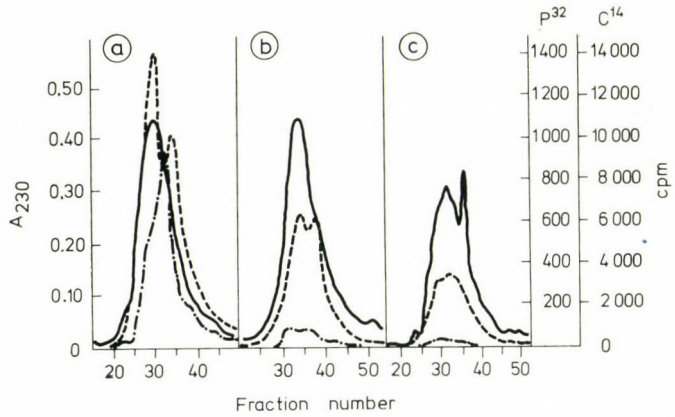

Fig. 3. Effect of nuclease treatment on the elution profile of informofer on a Bio-Gel A-5m column. Column size: 2.2×40 cm. —— A_{230}; — — (^{32}P)-orthophosphate, cpm; — · — · — (^{14}C)-orotate, sodium salt, cpm. *a*, control, without nuclease. The sample was incubated at room temperature for 20 min, without the addition of exogenous enzyme. *b*, the sample was incubated with 20 μg/ml of pancreatic RNAse I for 20 min at room temperature. *c*, the sample was treated as *b*, but the incubation mixture also contained 25 μg/ml DNAse

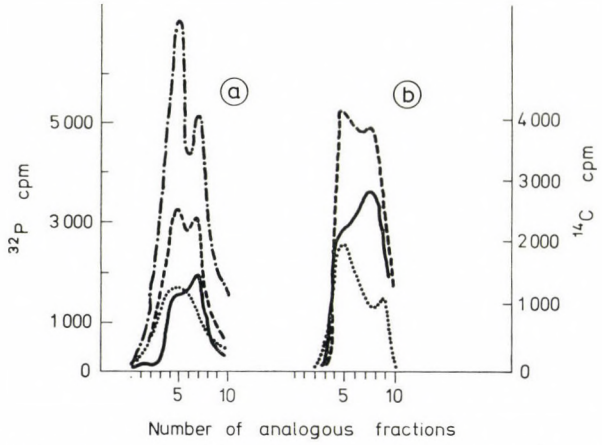

Fig. 4. Differential elution pattern of standard chromatographies of nuclease treated purified 30 *S* particles. (Bio-Gel A-5m column, 2.2×40 cm). *a*, radioactivity derived from (^{32}P)-orthophosphate. — · — · — control, without enzyme treatment; — — residual activity after RNAse treatment; · · · · · · residual activity after RNAse and DNAse treatment; ——— the difference between the last two radioactivity patterns, i.e. radioactivity liberated on the combined effect of RNAse and DNAse. *b*, radioactivity derived from (^{14}C)-orotate. — — residual activity after RNAse treatment; residual activity after combined treatment with RNAse and DNAse; ——— the difference between the last two, i.e. the effect of combined enzyme treatment

conditions (the same amount of the same starting material, standardized chromatography). Fig. 4 shows the differences between the analogous fractions of samples treated with RNAse and with RNAse + DNAse, as well as the ^{14}C and ^{32}P radioactivities, i.e. the radioactivity solubilized only by the combined attack of the two enzymes.

The elution pattern of rat liver cytosal particles prepared in the presence of RNAse inhibitor (Roth, 1958) is heterogeneous (Samarina et al., 1968). Treatment with RNAse or with RNAse + DNAse results in the decomposition of the complexes; the chromatographic picture is analogous to those of Fig. 3b and c.

Discussion

The results presented in this paper do not prove that all 30 *S* particles or polyparticles contain DNA, but there seem to exist species in which DNA plays a structural role. Samarina et al. (1967) have shown in their already classical work that DNAse-treatment does not alter the sedimentation pattern of 30 *S* particles, moreover, that a considerable part of labelled RNA remains bound to the particles even after treatment with RNAse. Part of the activity is due probably to the polyadenylic sequences that are just being synthesized in the 30 *S* particles (Niessing, Sekeris, 1972), but one should bear in mind that in the labelling system used by us the purine nucleotides are only labelled with orthophosphate. On the other hand, polyadenylic acid is resistant not only to pancreatic RNAse but also to DNAse. The susceptibility of the complexes to the combined attack of RNAse and DNAse allows one to make two assumption. First, DNA may be a structural constituent of the individual complexes; second, part of the RNA bound to the informofer particles may be present in the form of natural DNA–RNA hybrids.

The existence of nuclear particles containing DNA, RNA and protein has already earlier been demonstrated (Pogo, 1968; Puvion et al., 1969) but it is difficult to assess whether the formations described were related to the informofer particles.

The study of DNA bound to the informofer is in progress in our laboratory.

Thanks are due to Drs O. P. Samarina and G. P. Georgiev, and to Prof. A. Tigyi and Dr L. Komáromy for their continuous help and criticism. The devoted technical assistance of Mrs K. Herner and Mrs M. Schäffer is greatly appreciated.

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The Heterogeneity of Informofer

V. Effect of KCl on the Various Types of 30 *S* Nuclear Particles Containing dRNA

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The stability of dRNA-containing ribonucleoproteids (30 *S* particles) isolated from rat liver cell nuclei was studied in KCl solutions of various concentrations which did not effect complete dissociation. It was shown that the "0.1 M 30 *S* particles" described earlier, which are presumably in charge of the nucleocytoplasmic transport of mRNA, were decomposed into RNA and informofer (protein component) at a higher ionic strength than were the "0.3 M 30 *S* particles". The latter are mainly composed of dRNA segments degraded in the cell nucleus. The degree of fragmentation of dRNA released from the "0.3 M particles" was greater than that of mRNA bound to the "0.1 M 30 *S* particles".

Introduction

The dRNA synthesized in the cell nucleus forms complexes with a globular protein component, the informofer, which can be extracted from the cell nucleus by physiological salt solution at mildly alkaline pH. In the extract one can find either polysome-like complexes, or the so-called nuclear 30 *S* particles, depending on whether the extracting solution contained RNAse inhibitor or not (Samarina et al., 1968).

We have earlier described that the nuclear 30 *S* components dissociate to dRNA and protein at relatively low salt concentrations in a reversible manner (Samarina et al., 1967a, b).

Furthermore, it has been shown that on further extraction of the cell nucleus with buffer containing 0.3 M NaCl instead of 0.1 M, an additional appreciable amount of dRNA-containing particles can be obtained. This fraction also contains 30 *S* particles, but their RNA content is lower than in the "0.1 M particles" if extraction is performed in the absence of RNAse inhibitor and the protein composition of the particles is also different (Molnár, Juhász, 1972). The two kinds of particles are morphologically different (Komáromy et al., 1973) and differ in their degradation kinetics, too (Molnár et al., 1973). We have assumed on the basis of our investigations and data in the literature that the "0.1 M particles" (extracted with buffer containing 0.1 M NaCl) are responsible for the transport of mRNA, whereas the "0.3 M particles" form complexes with dRNA segments degraded in the nucleus (Drews, 1969; Molnár, Juhász, 1972; Molnár et al., 1973; Juhász, Molnár, 1973).

In the present work the properties of "0.1 M" and "0.3 M particles" were studied at KCl concentrations which did not effect the complete dissociation of the particles. It is shown below that the dissociation of 30 *S* particles commences already in 0.35 M KCl, but the extent of dissociation and the degree of fragmentation of liberated RNA are different with the "0.1 M" and "0.3 M particles".

Materials and methods

Most chemicals were purchased from Reanal (Budapest) and were of reagent grade. 2-(^{14}C) orotic acid (specific activity 10.35 mCi/mmole) and carrier-free $NaH_2{}^{32}PO_4$ were obtained from the Isotope Institute of the Hungarian Academy of Sciences.

The sucrose-containing solutions were shaken with diethylpyrocarbonate (Fluka) before use to remove possible RNAse contaminations.

In the experiments male CFY rats weighing about 200 g, from our own breed, were used. Before the experiments the animals starved for 24 hours. The rats were given 10 to 15 μCi sodium (^{14}C)-orotate 25 minutes before killing, or 1 mCi of $Na_2H^{32}PO_4$ two hours before killing, both intraperitoneally. The animals were killed in ether anaesthesia, the livers were perfused with ice-cold physiological saline, homogenized in a glass-teflon Potter-Elvehjem type homogenizer in 5 volumes of 0.5% sodium β-glycerol-phosphate–2.2 M sucrose solution, then the nuclear fraction was isolated as described previously (Samarina et al., 1967). All operations were carried out between 0 and 4 °C.

The nuclear fraction was extracted twice with STM II (0.1 M NaCl, 0.01 M Tris HCl, 0.001 M $MgCl_2$, pH 7.9), then twice with STM III (the same as STM II, but 0.3 M instead of 0.1 M NaCl) buffers (Molnár, Juhász, 1972).

The extracts were centrifuged in a sucrose gradient which also contained 0.01 M triethanolamine and 0.001 M $MgCl_2$, pH 7.5, and the required amount of KCl. The samples were layered on top of a 32 ml 15 to 30% (w/v) sucrose gradient and were centrifuged for 14 hours in a Beckman Model L2-65B preparative ultracentrifuge (rotor SW 27) at 24 000 r.p.m. and 3 °C. In the experiments with particles already purified by gradient fractionation, the appropriate fractions were pooled and dialyzed for 4 to 5 hours against STM II containing triethanolamine (pH 7.5), to remove excess of sucrose.

For CsCl isopycnic density gradient centrifugation the material to be tested was fixed with 4% neutralized formaldehyde, dialyzed against 0.005 M phosphate buffer, pH 7.2, containing 1% formaldehyde, and then spun through a 5 ml preformed CsCl gradient in the 3×5 ml rotor of a Janetzki VAC 60 ultracentrifuge, at 36 000 r.p.m. and 10 °C for 20 hours (Molnár, Komáromy, 1972).

The fractions of the gradient were assayed for optical density at 260 nm, then after the addition of a suitable amount of rRNA as carrier the acid-insoluble material was washed into a cellulose nitrate filter (Synpor 2), dried with ethanol, and the radioactivity was measured in a Gamma liquid scintillation spectrometer (Molnár et al., 1973). The scintillation cocktail contained 100 mg POPOP and 4 mg PPO in 1000 ml toluene.

Results and discussion

It was shown in our earlier experiments that the buoyant density of "0.3 M particles" was always lower than that of "0.1 M particles" provided that the extracting solutions did not contain RNAse inhibitor. Furthermore, the increase of NaCl concentration to 0.3 M in the "0.1 M nuclear extract" did not alter the protein composition of the particles (Molnár, Juhász, 1972).

As in the present experiments we also dealt with the mixture of "0.1 M" and "0.3 M" nuclear extracts, it was necessary to check whether or not 0.3 M NaCl concentration changed the RNA/protein ratio in the "0.1 M particles". To this end NaCl was added up to 0.3 M final concentration to an aliquot of "0.1 M nuclear extract" prepared without RNAse inhibitor, the mixture was allowed to stand in ice-water for half an hour and then layered on top of a sucrose density gradient and centrifuged in the usual way. The sedimentation pattern, not documented here, was identical with that of the control "0.1 M particles".

The fractions of the gradient containing the particles were pooled, fixed with formaldehyde and then dialyzed against 0.005 M phosphate buffer containing 1% formaldehyde. The material thus obtained was mixed with an aliquot of "0.3 M particles" extracted without RNAse inhibitor and fixed and dialyzed as above, and the mixture was subjected to CsCl equilibrium density gradient centrifugation. Since the "0.1 M particles" carried radioactivity derived from (^{32}P)-orthophosphate, whereas the "0.3 M particles" were obtained from animals

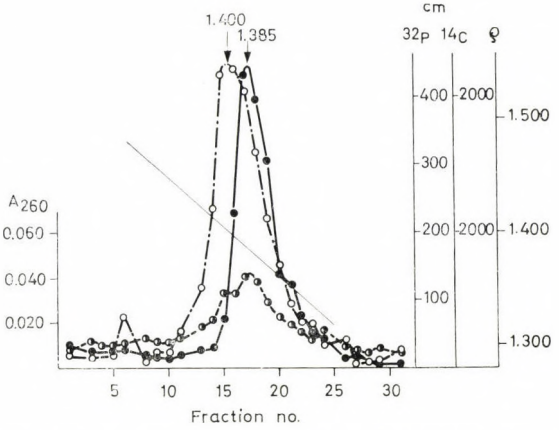

Fig. 1. CsCl density gradient equilibrium centrifugation of "0.1 M" and "0.3 M" nuclear 30 *S* particles. In the "0.1 M nuclear extract" NaCl concentration was adjusted to 0.3, then from this, and from the "0.3 M extract", the 30 *S* particles were isolated by sucrose gradient centrifugation. After fixation (with 4% neutralized formaldehyde) of the fraction found in the 30 *S* zone, the sample was dialyzed against 0.005 M phosphate buffer, pH 7.3, containing 1% formaldehyde. The preformed CsCl gradient ($\rho = 1.28-1.52$ gcm^{-3}) was made up with the mixture of the two particle suspensions. Centrifugation was carried out in 3×5 hours, at 36 000 r.p.m. and 10 °C. Three-drop fractions were collected. The density of every fifth fraction was determined on the basis of refraction. ◑---◑: A_{260}; ●—●: ^{14}C-radioactivity ("0.3 M particles"), dpm; ○—·—○: ^{32}P radioactivity ("0.1 M particles"), dpm

which were administered (^{14}C)-orotate before the experiment, the position of the two types of particles could be readily located within the same gradient (Fig. 1). It is seen that the elevation of NaCl concentration to 0.3 M in the "0.1 M extract" did not influence the buoyant density of these particles.

The difference between the buoyant densities of the two particles shown in Fig. 1 was consistently observed, inasmuch as for the "0.1 M particles" the value was about 0.010 – 0.020 gcm^{-3} higher. This difference was not affected by the fact that the RNA of which particle was labelled with ^{32}P (2 hours of incubation) and which with ^{14}C (25 min of incubation). It is also seen that this difference is not the result of the different ionic strengths during extraction. We rather think that the difference is due to the partial loss of RNA from the "0.3 M particles" during manipulation, since if the extracting solutions contained RNAse inhibitor the buoyant densities of both particles equaled 1.40 gcm^{-3} (Molnár, Juhász, 1972).

In the subsequent experiments the nuclear extracts were centrifuged or the 30 *S* particles were resuspended, in 15 – 30% sucrose gradients which contained KCl instead of 0.1 M NaCl in the buffer solution.

In the experiment to be described "0.1 M" and "0.3 M" nuclear extracts were mixed. The former was obtained from the liver of animals that were administered 15 μ Ci (^{14}C)-orotate 25 minutes before killing, whereas the latter was prepared from animals that were given 1 mCi ^{32}P two hours before starting the experiments. The mixture of extracts was layered onto sucrose gradients containing 0.35, 0.40, 0.45, and 0.50 M KCl (Figs 2b, c, d and e, respectively) and centrifuged in the usual way. The advantage of using the mixture of the two kinds of particles was that the differences could be evaluated within the same gradient, under identical conditions.

In the next set of experiments we followed the above protocol except that not the extracts but the particle preparations isolated by gradient centrifugation were mixed, later the excess of sucrose was removed by dialysis.

As the sedimentation pattern of these runs was essentially the same as obtained with the mixture of extracts, only the patterns of gradients containing 0.4, 0.6 and 0.7 M KCl are documented, along with the control (Figs 3a, b, c, and d).

The experiments show that the particles are markedly damaged already in 0.35 M KCl (Fig. 2b) and on increasing KCl concentration the dissociation of 30 *S* particles is enhanced and at 0.7 M concentration (Fig. 3c) attains the final value (Samarina et al., 1967a, 1967b). However, the behaviour of the two types of particles towards increasing KCl concentration is different. In the case of "0.1 M particles" there is a pronounced peak already at 0.35 M KCl concentration corresponding to the 22 – 25 *S* zone (Fig. 2b, fractions 19 – 21), which predominates even at 0.45 M KCl concentration (Fig. 2d). The 20 – 22 *S* peak is considerable even in 0.50 M KCl (Fig. 2e, fractions 21 – 23), and disappears completely only at higher KCl concentrations (Fig. 3c and d). On the other hand, with the "0.3 M particles" there is no definite peak already at 0.45 M KCl (Fig. 2d), and the profile characteristic of complete dissociation appears at 0.50 M KCl concentration (Fig. 2e). The other significant difference is that whereas only a

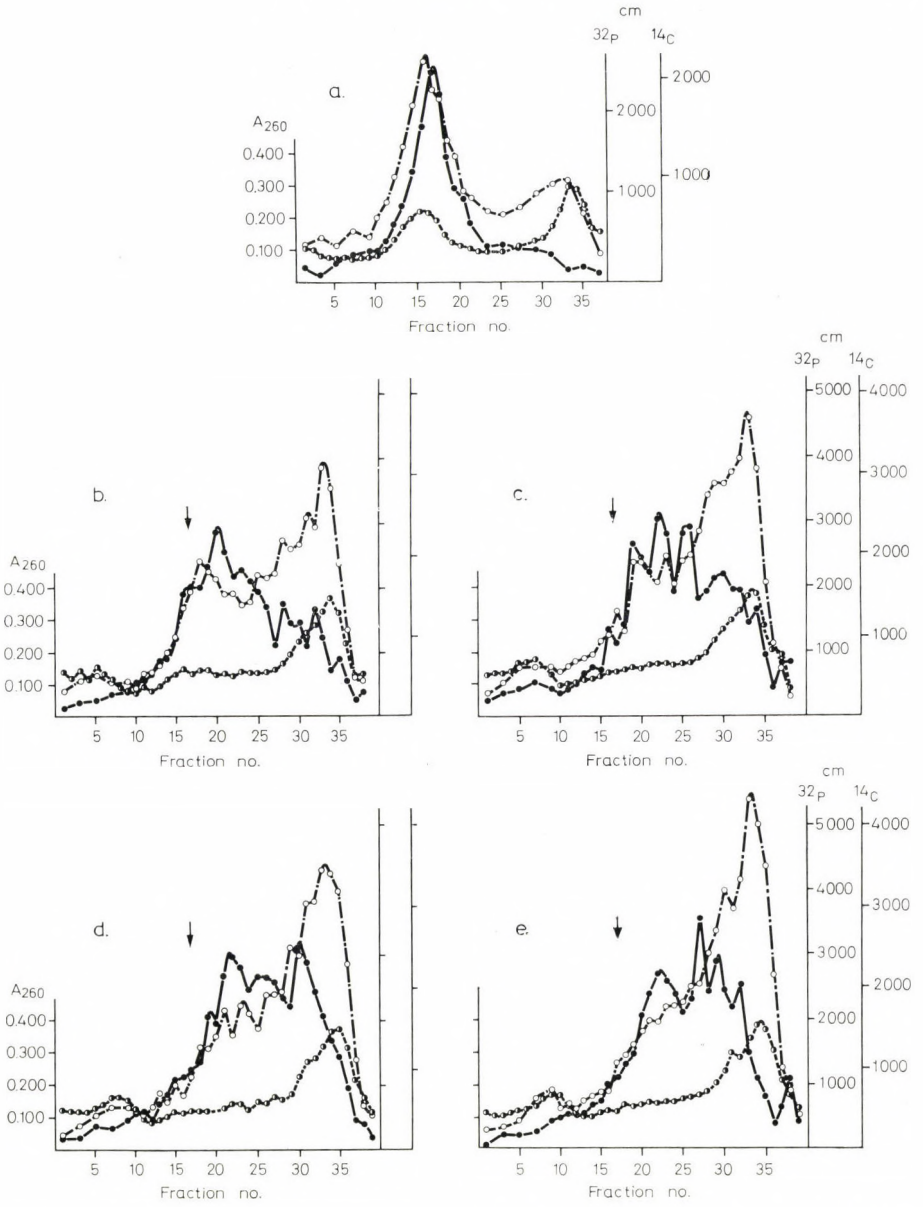

Fig. 2. Sedimentation patterns of a mixture of "0.1 M" and "0.3 M" nuclear extracts in linear sucrose density gradients containing various amounts of KCl. 5.5 ml "0.1 M extract" and 4.5 ml "0.3 extract" were mixed and from the mixture 2 ml was layered on top of a 32 ml 15–30% (w/v) sucrose gradient. In addition to sucrose the gradient contained 0.001 M $MgCl_2$, 0.01 M triethanolamine, pH 7.5, and KCl in the following concentrations (M). a) 0.1 M; b) 0.35; c) 0.40; d) 0.45; e) 0.50. Sedimentation was carried out in the SW 27 rotor of a Beckman Model L2-65B preparative ultracentrifuge for 14 hours at 24 000 r.p.m. and 3 °C. Thirteen-drop fractions were collected. ◑- - -◑: A_{260}; ●—●: ^{14}C radioactivity ("0.1 M particles") dpm; ○—·—○ : ^{32}P radioactivity ("0.3 M particles") dpm. The arrow indicates the position of 30 *S* zone. Direction of sedimentation: from right to left

negligibly small portion of the radioactivity of "0.1 M particles" can be found in the zone lighter than 10 *S* even in 0.5 M KCl (Fig. 2e), the majority of label of "0.3 M particles" is found in the 5–6 *S* zone already in 0.35 M KCl (Fig. 2b), which later considerably increases.

If the dissociation of "0.1 M" and "0.3 M" particles was carried out separately, the pattern obtained was essentially the same as above.

In contrast to other nucleoproteids (such as ribosomes), with the nuclear ribonucleoproteids containing dRNA the increase of ionic strength does not result in the release of proteins from the complex but rather of RNA, while the protein moiety, the informofer, remains intact as free informofer complex (Lukanidin et al., 1972). Moreover, on the electron micrographs taken at 0.5 M KCl concentration particles can be discerned which are linked to filaments of the size and appearance of RNA (Komáromy, personal communication). Thus in our case the material sedimenting in the 15–25 *S* zone of the gradient and derived from "0.1 M particles" may represent particles which are already partly disintegrated but the RNA is still in connection with the informofer, since the RNA itself cannot be larger than 8–9 *S* (Samarina et al., 1968), whereas the protein part, the informofer, remains intact (Lukanidin et al., 1972).

On increasing the ionic strength more and more informofer is deprived of the RNA, the latter being found then in the 8–10 *S* zone (Figs 2d, e, 3c), or its further degradation products in the lighter fractions (Fig. 3d).

It has already been shown that the RNA of "0.3 M particles" is much more sensitive to the effect of RNAse than is the RNA of "0.1 M particles", and the "0.3 M particles" prepared in the absence of RNAse inhibitor contain *ab ovo* less RNA than "0.1 M particles" isolated in the same way (Molnár, Juhász, 1972). The fact that the RNA fragments released on increasing ionic strength are relatively short segments could be explained by assuming that the RNA of "0.3 M particles" has already been fragmented when it was still bound to the particle. This is supported by the fact that the dissociating agent exerted its effect on both materials under the same conditions, yet only in the case of "0.3 M particles" resulted in the production of large amounts of short fragments. If the "0.1 M particles" also contained markedly fragmented RNA, then these fragments should form rather stable complexes with the informofer, as little radioactivity is found in the 4–6 *S* zone even at 0.5 M KCl concentration in the case of these particles.

The first possibility is more probable since in most gradients the RNA of "0.3 M particles" could be found in large amounts in the 4–6 *S* zone. At any rate it can be unequivocally established that at KCl concentrations not effecting complete dissociation the behaviour and stability of "0.1 M" and "0.3 M" particles are different.

There is another interesting phenomenon: in the 40–45 *S* zone appreciable amounts of material may appear on increasing the ionic strength (Fig. 3c and e), which contain little radioactivity, especially ^{14}C. We tend to believe that this fraction comprises aggregated free informofer, as the same phenomenon was observed on the effect of RNAse treatment and morphologically there was

indeed an aggregate (Molnár, Komáromy, 1974). Experiments to elucidate this observation are under way in our laboratory.

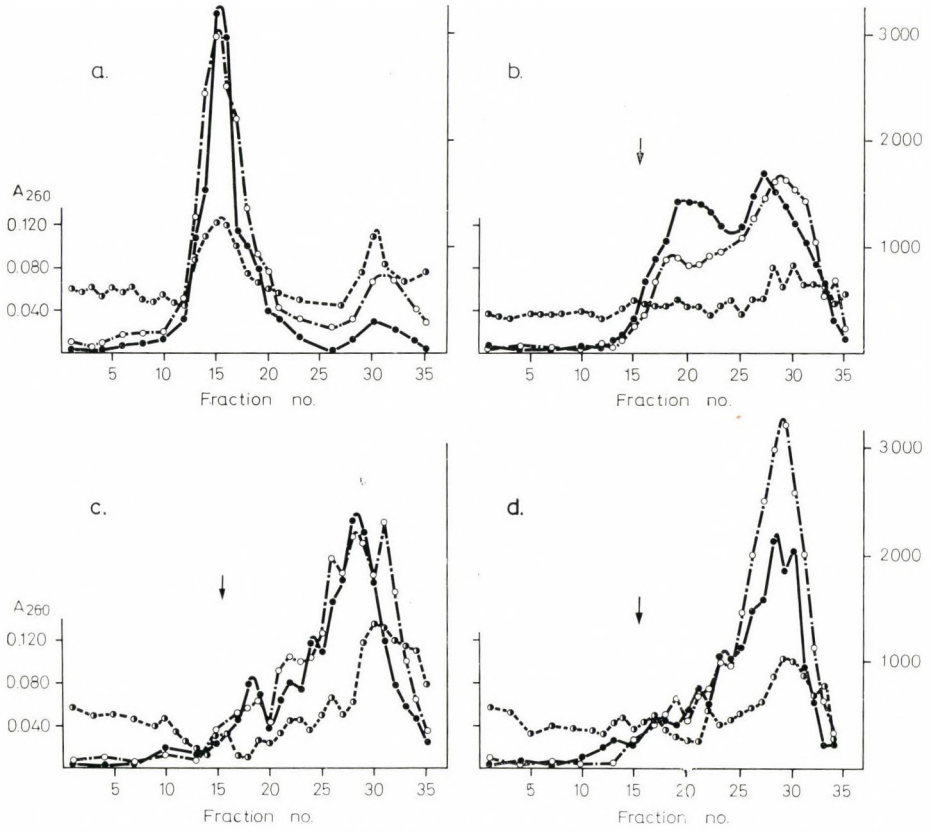

Fig. 3. Re-sedimentation of a mixture of purified "0.1 M" and "0.3 M" nuclear 30 *S* particles in sucrose gradient containing KCl. The 30 *S* particle preparations purified by sucrose density gradient centrifugation after dialysis were mixed and equal volumes (2.5 ml) were layered on top of 32 ml 15—30% (w/v) sucrose gradients which were made up in the buffer described in Fig. 2 and also contained the following KCl concentrations (M): a) 0.1; b) 0.4; c) 0.6; d) 0.7. The conditions of centrifugation and the symbols are the same as in Fig. 2. Fractions of 15 drops were collected. The arrow indicates the position of 30 *S* zone. Direction of sedimentation: from right to left

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Relationship between Primary Structure and Lipolysis Stimulating Activity of β-lipotropic Hormone

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The structural basis of the lipolysis stimulating activity of pig β-lipotropic hormone was investigated by comparing fragments that represent different portions of the amino acid sequence. The activity was measured by lipolysis in vitro of rabbit adipose tissue and was characterized in term of the log-dose response curve, the minimal effective concentration and competition with β-lipotropic hormone. All the fragments had smaller activity than β-lipotropic hormone. Peptides containing sequence 44—60 proved to be most active. From the differences between the fragments we concluded that besides the sequences common with other lipolytic hormones there are further active sites in the peptide chain and these contribute to the lipolysis stimulating activity of β-lipotropic hormone.

Introduction

Li et al. (1965) were the first to report the isolation of a new lipotropic peptide from sheep hypophysis and they named it β-lipotropic hormone (β-LPH). This peptide consisting of 90 amino acids was the object of investigations aimed at the clarification of biological properties (Lohmar, Li, 1968; Gattereau et al., 1970; Desranleau et al., 1972). However, chemically characterizing β-LPH obtained from pig hypophysis we observed that its primary structure differed from that of the sheep hormone to a considerable extent; the chain consists of 91 residues and amino acid replacements can be found at fourteen positions (Cseh, 1969; Gráf et al., 1971). Similar observations have recently been made by Pankov and Yudaev (1972). These species variations prompted us to reinvestigate the relationship between peptide structure and lipolysis stimulating activity in the case of pig β-LPH.

In our laboratory the various fragments of β-LPH and of its 1—58 portion (γ-LPH) were obtained by plasmic, tryptic, and chymotryptic digestion as well as by cyanogen bromide cleavage. Some of these fragments seemed to be suitable for the above studies. In addition, Dr Medzihradszky placed a synthetic peptide at our disposal, which was identical with portion 44—60 of β-LPH.

Abbreviations: β-LPH: β-lipotropic hormone; γ-LPH: γ-lipotropic hormone; α-MSH: α-melanotropic hormone; β-MSH: β-melanotropic hormone; FFA: free fatty acid.

Materials and methods

The β-LPH was isolated from pig adenohypophysis as described earlier (Gráf, Cseh, 1968). Its amino acid sequence is shown in Fig. 1. The fragments 1 – 79, 1 – 60, 1 – 51 and 1 – 46 were obtained by plasmic digestion as follows. β-LPH was hydrolyzed with Actase (Cilag Chemie) in 0.05 M ammonium acetate buffer, pH 7.4, at 37 °C for 2 hours with an enzyme to peptide ratio of 1 : 20 (w/w). Then the fragments were filtered through Biogel P-10 and subse-

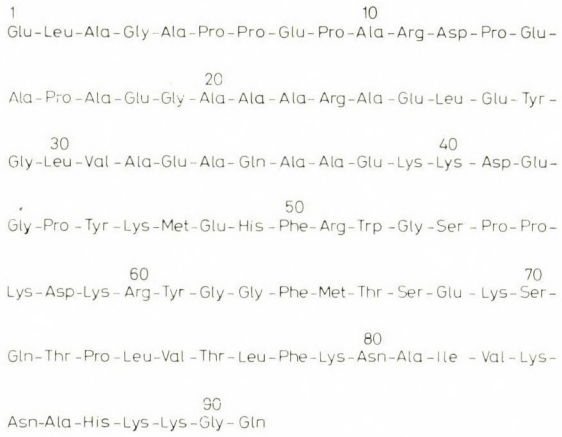

Fig. 1. The amino acid sequence of pig β-LPH (Gráf et al., 1971)

quently isolated by CM-cellulose chromatography (Gráf, 1972). γ-LPH was prepared from pig hypophysis as reported earlier (Gráf et al., 1969). Fragment 48 – 58 was obtained by the cyanogen bromide reaction of γ-LPH (Gráf et al., 1970). Fragments 47 – 51 and 29 – 45 derived from the tryptic and chymotryptic digestion of β-LPH (Gráf et al., 1971). Homogeneity of the fragments was checked by end-group analysis and gel electrophoresis, their other properties are given in the literature cited.

The peptides were tested for lipolysis in vitro on the renal adipose tissue of male rabbits (body weight 3 kg) kept on normal food. Fat tissue pieces of 200 mg were incubated with different amounts of peptide in 3 ml of Krebs-Ringer phosphate buffer, pH 7.4, at 37 °C for one hour. The control sample did not contain peptide. The reaction was stopped by adding 10 ml of Dole's reagent and after homogenization the free fatty acids were titrated (Dole, Meinertz, 1960). The lipolytic activity is shown by the increase of the quantity of fatty acid obtained in the presence of the peptide tested and is expressed in μmoles FFA/g tissue/hour units.

Results

Plotting the formation of FFA versus peptide concentration in a semilogarithmic diagram we obtained a sigmoid curve in a number of experiments performed with pig β-LPH and its active fragments. The data of such a character-

istic experiment are shown in Fig. 2. It can be seen that the curves are shifted along the abscissa in different degrees, which indicates the relative effectiveness in decreasing order as follows: β-LPH, fragment 1–60, peptide 44–60, γ-LPH and fragment 48–58. The curves of fragment 1–60 and γ-LPH have the same maximum and run parallel with the curve of β-LPH. The curves of peptides 44–60 and 48–58, however, have different courses.

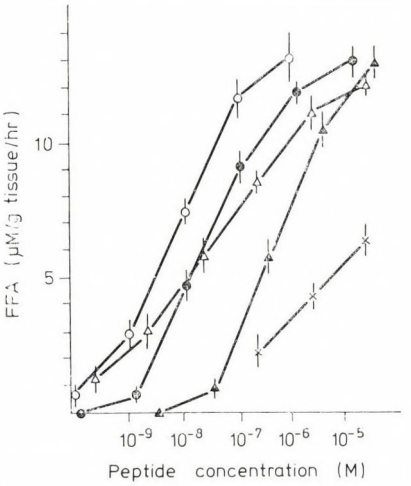

Fig. 2. The log-dose response curve of lipolytic activites of β-LPH and its fragments. Each point indicates the mean value obtained with five pieces of fat tissue from a single rabbit. The standard error is designated by vertical bars. ○, β-LPH; ●, fragment 1–60; △, peptide 44–60; ▲, γ-LPH; x, fragment 48–58

As a measure of the quantitative comparison of lipolysis-stimulating activity of the examined peptides the minimal effective concentration was chosen. According to Stetten (1956) the log-dose response curve expresses the relationship between the number of receptors relevant to the hormone and the biological effect, wherein the affinity of the hormone to the receptor is indicated by the minimal effective concentration. The latter was calculated from the middle part of the log-dose response curve, extrapolated to the formation of 1 μmole of FFA/g tissue/hour. The results of experiments performed on the adipose tissues of five rabbits are summarized in Table 1.

Although the susceptibility of adipose tissue varied by animals, a certain relationship between the lipolysis-stimulating activities of β-LPH and its fragments could be observed with all rabbits. β-LPH was found to be most active; its minimal effective concentration is two or three times as small as that of peptide 44–60. Furthermore, it can be established that fragment 1–79 is five times, peptide 1–60 is three times and γ-LPH is 134 times less active than β-LPH. Fragment 1–51 and its portions – below 10^{-6} M concentration – proved to be inactive.

With the inactive peptides we performed further experiments by a method analogous to the detection of hapten inhibition (Kabat, Mayer, 1958). β-LPH

Table 1

The lipolysis-stimulating activity of β-LPH and its fragments in term of minimal effective concentration

Peptide	Minimal effective concentration (10^{-10} M)				
	1	2	3	4	5
β-LPH	3.5	9.1	3.5	5.1	10.0
Fragment 1—79	43.7	25.2	16.0	23.0	46.0
Fragment 1—60	21.2	15.1	10.2	—	30.3
γ-LPH	156.2	193.7	59.4	134.4	125.0
Fragment 1—51	—	>10 000			
Fragment 1—46	>10 000		—	>10 000	
Peptide 44—60	6.4	18.5	12.3	—	18.5
Fragment 48—58	330	—	620	500	483
Fragment 47—51	—	—	>10 000		—
Fragment 29—45	—	>10 000	—	>10 000	—

and various amounts of the peptide in question were added simultaneously to the adipose tissue and the magnitude of lipolysis was measured. It could be seen that fragment 47—51 was able to prevent the formation of FFA if the concentration of β-LPH was not so high to elicit maximal response (Fig. 3). Inhibition of lipolysis depends on the concentration ratio of the two peptides.

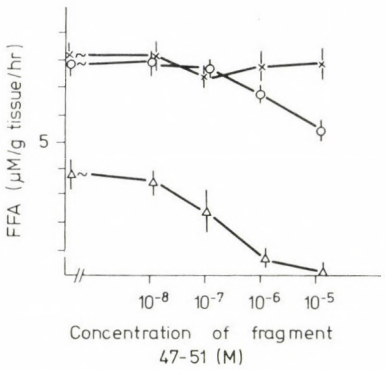

Fig. 3. The effect of fragment 47—51 on lipolysis by β-LPH. The concentration of β-LPH: 2×10^{-7} M (×), 2×10^{-8} M (○), and 2×10^{-9} M (△)

It is remarkable that the molar concentration of fragment 47–51, relative to β-LPH, should be two orders of magnitude higher to decrease lipolysis to one half. Such an inhibitory effect was not found with either fragment 1–46 or 29–45.

Discussion

Our results show that several fragments having lipolysis-stimulating activity can be obtained by proteolysis of β-LPH. Cleavage with plasmin in the C-terminal portion of the polypeptide chain proved to be especially successful as the fragments obtained in this way allowed us to study the role of certain residues near the C-terminus.

The earlier studies on the relationship between primary structure and lipolytic activity of sheep LPH were based upon the comparison of biological activities of "natural fragments" (γ-LPH, β-MSH), and partly of fragments obtained by cyanogen bromide cleavage (Chrétien, Li, 1967). On this basis it was suggested that the biologically active center of β-LPH is constituted by sequence 41–58, which is identical with the primary structure of β-MSH.

Comparing β-MSH, α-MSH, ACTH and arginine-vasopressin, Rudman (1970) assumed that a common portion: Tyr.a.b.Glu.c.d.Arg*, formed the structural basis of lipolytic activity.

In the primary structure of pig β-LPH the portion (sequence 45–51) in which the relative position of Tyr, Glu and Arg are the same as in sheep β-LPH and other lipolytic peptides of the hypophysis can also be found. Among the peptides studied by us, the smallest that contained the portion Tyr.a.b.Glu.c.d.Arg* was fragment 44–60. Its high lipolysis-increasing activity lends support to accept as active center the portion suggested by Rudman in the case of pig β-LPH. Our experiments with fragment 47–51 reveal, however, new details concerning the function of this active center. Namely, the competition with β-LPH can be explained by assuming that Glu-48 and Arg-51 play a role in binding to the receptor but further amino acids are required for the induction of lipolytic process itself. The significance of amino acids linked to the C-terminal of fragment 47–51 is stressed by the high activity of fragment 48–58.

Similar conclusions can be drawn from the experiments performed on certain synthetic ACTH fragments (Tanaka et al., 1962).

The activity of fragment 48–58 still proved to be two orders of magnitude lower than that of β-LPH, which shows that for the high effectivity of the latter further parts of the polypeptide chain are needed. The observation that fragment 1–60 obtained by plasmic digestion is more active than γ-LPH leads to the conclusion that residues Lys-Arg (59–60) play a role in increasing biological activity. The importance of another portion is shown by the difference found between the biological activities of β-LPH and fragment 1–79. From this we conclude that the increase of activity must be attributed to certain so far uniden-

* Positions a, b, c, and d can be occupied by various amino acids.

tified groups within sequence 80–91. Therefore it appears that while in the case of ACTH joining of fragment Lys-Lys-Arg to the N-terminal fragment 1–14 (Bajusz, Medzihradszky, 1967) complete the lipolytic activity, the presence of further residues is required in the C-terminal part of β-LPH.

It is to be mentioned, however, that Chrétien and Li (1967), comparing the activities of β-LPH and fragment 1–65 obtained by cyanogen bromide cleavage of sheep β-LPH, did not observe any increasing effect by the C-terminal. They found, in addition, that sheep β-MSH is more active than γ-LPH by at least one order of magnitude; this means that amino acids linked to the N-terminus of portion 41–58 in β-LPH decrease biological activity. Behaviour of fragment 1–51 in our experiments seems to be in good agreement with this: although it contains the active center suggested by Rudman (1970), it has no activity. Comparing peptide 44–60 and fragment 1–60 with fragment 48–58 and γ-LPH we did not find any such differences between the activities from which the inhibitory role of fragment 1–43 or 1–47 could have been deduced. In these cases the presence of the N-terminal portion manifested itself in a change of the slope of the dose response curve. Thus, it seems that no inhibitory effect of the N-terminal of lipotropin is observed if further amino acids are linked to the C-terminal of the active centrum.

Our experiments were performed only on pig β-LPH and its fragments. The amino acid sequence of pig and sheep β-LPH's is identical between residues 43–82. Since most of the residues responsible for lipolysis-stimulating activity are located here, we assume that the effect on the metabolism of adipose tissue is essentially the same in the two species. This is supported by our earlier experiments according to which the activities measured on rabbit and rat fat tissues were very similar (Cseh, 1969).

Furthermore, the observation that the antiserum against sheep β-LPH gives cross-reaction with pig β-LPH is also in accordance with this conclusion (Desranleau et al., 1972).

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The Effect of Quinacrine on Induced β-galactosidase Synthesis in *Escherichia coli*

(Preliminary Communication)

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(Received July 3, 1973)

Quinacrine mustard has been applied as a chromosome stain producing specific individual fluorescent bands on human metaphase chromosomes (Caspersson et al., 1968). Later it was demonstrated that quinacrine itself possessed the same specificity as its mustard derivative (Vosa, 1970).

Proflavine, another acridine derivative, has been successfully applied to arrest the transcription of the *lac* operon of *Escherichia coli* (Perlman, Pastan, 1968; Pastan, Perlman, 1969; Aboud, Burger, 1970). These results prompted us to test quinacrine in blocking induced β-galactosidase synthesis.

Here we report that quinacrine specifically inhibits the transcription of the *lac* operon in *E. coli*, i.e. the transcription of the *lac* operon is inhibited by quinacrine at such low concentrations which do not influence overall RNA and protein synthesis.

E. coli K12 wild type cells were grown in mineral salts + glycerol minimal medium (Schlammadinger, Szabó, 1971). An overnight culture of the cells was diluted in fresh, prewarmed medium to $A_{570} = 0.075$ and cultivated in a reciprocal water bath shaker (Gallenkamp IH-350), at 37 °C. At $A_{570} = 0.300$, i.e. in the logarithmic phase, the cells were induced with 5×10^{-4} M methyl-β-D-thiogalactoside (Serva). Aliquots of this induced culture were pipetted after 15 minutes successively to Erlenmeyer flasks containing Quinacrine (Sigma) in the required concentration. The estimation of β-galactosidase activity was performed as described earlier (Schlammadinger, Szabó, 1971). One enzyme unit equaled 1 μmole of *o*-nitrophenyl-β-D-galactopyranoside (Fluka) hydrolyzed per ml bacterial suspension* per hour, at 37 °C.

The overall RNA and the net protein syntheses were followed by the incorporation of (^{14}C)-uracil (0.15 μCi/ml) and (^{14}C)-L-leucine (0.07 μCi/ml), respectively, into cold 5% trichloroacetic acid-insoluble fractions. The precipitate was collected on membrane filters (Synpor 6, VCHZ Synthesia) and the radioactivity was measured in toluene + PPO + POPOP scintillator coctail in a Packard Tri-Carb liquid scintillation spectrometer.

* Prepared under standard conditions.

Fig. 1 summarizes the results. It can be seen that 50 μg/ml quinacrine strongly inhibits the induced β-galactosidase synthesis. The degree of inhibition becomes lower as the concentration of quinacrine diminishes. The two lowest concentrations tested still inhibit enzyme synthesis, without decreasing the incorporation of labelled uracil and L-leucine. This striking divergence of β-galactosidase synthesis on one hand, and the overall RNA and protein synthesis on the other suggested us that the *lac* operon of *E. coli* chromosome may contain a region which has increased affinity to quinacrine.

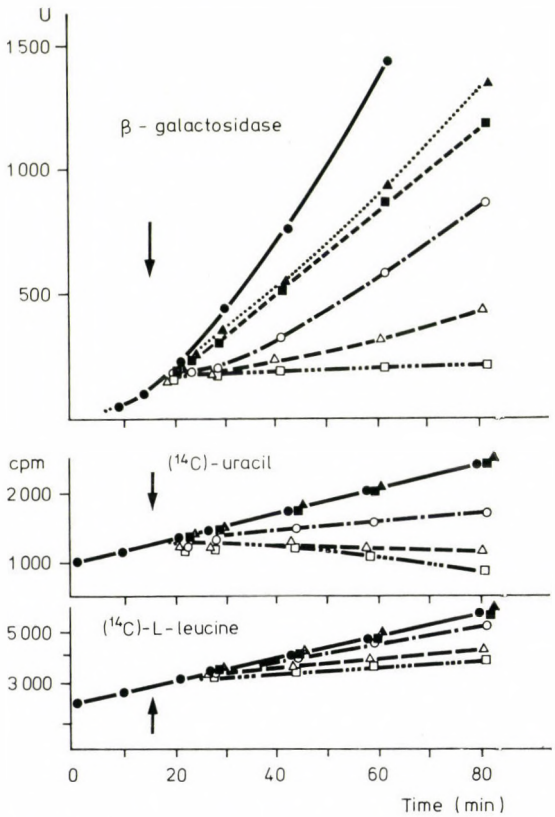

Fig. 1. Synthesis of induced β-galactosidase, incorporation of (^{14}C)-uracil and (^{14}C)-L-leucine at different quinacrine concentrations. Induction at 0 minute. Start (arrow) of successive addition of quinacrine at the 15th minute. (The whole procedure required about 60 seconds.) ●—● control; ▲·····▲ 3.12 μg/ml; ■----■ 6.25 μg/ml; ○—·—○ 12.5 μg/ml; △----△ 25 μg/ml; □—·—□ 50 μg/ml

It is known (Kepes, 1969) that the rate of transcription on the *lac* operon depends on the number of mRNA molecules initiated per unit time. The RNA polymerase binds to the *lac* promoter gene in the presence of cyclic adenosine-3′,5′ monophosphate (cAMP) and cAMP receptor protein (DeCrombrugghe et al., 1971). We may interpret our results by assuming that quinacrine decreased the

rate of *lac* mRNA initiation, i.e. that quinacrine acts on the *lac* promoter gene. Furthermore, we know that both RNA-polymerase (Le Talaer, Jeanteur, 1971) and quinacrine (Weisblum, De Haseth, 1972) preferentially bind to A–T rich segments of DNA. Therefore we assume that the *lac* promoter gene of *E. coli* may be a segment of the bacterial chromosome rich in A–T base pairs.

Preliminary observations with another *E. coli* strain support our hypothesis. In the CA-8050 strain, which carries a promoter mutation (UV5), quinacrine equally inhibited β-galactosidase synthesis and the overall RNA and protein production. These findings, however, need further confirmation with different other mutant strains.

The authors wish to express their thanks to Dr M. D. Yudkin for the *E. coli* CA-8050 strain, to Dr J. Janeček for providing the (^{14}C)-L-leucine and membrane filters, to Dr J. Csongor for the radioactivity measurements and to Mrs Ibolya Szekeres for the skilled technical assistance.

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The Effect of D-tryptophan upon Induced β-galactosidase Synthesis in *Escherichia coli*

(Preliminary Communication)

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Certain amino acid analogues are potent inhibitors of protein synthesis. Such results were reported among others for 5-methyltryptophan and β-2-thienylalanine (Turnock, Wild, 1966), different phenylalanine analogues (Janeček, 1967) and about the incorporation of β-2-thienylalanine into β-galactosidase (Janeček, Rickenberg, 1964). Janeček investigated β-galactosidase synthesis in *Escherichia coli* in the presence of 7-azatryptophan (Janeček, Spižek, 1969) and ethionine (Spižek, Janeček, 1969). There are reports about the effect of norleucine (Kerwar, Weissbach, 1970; Richmond, 1962). Kepes (1969) described the use of 5-methyltryptophan as an analogue that inhibited β-galactosidase synthesis in *E. coli*.

In this paper we present evidence that D-tryptophan can also be applied as a protent inhibitor of induced β-galactosidase synthesis in *E. coli* cells and its effect is prevented or completely reversed by its natural stereoisomer, L-tryptophan.

E. coli K12 wild type cells were generally used. Cultivation of the cells and all other procedures were essentially the same as described previously (Schlammadinger, Szabó, 1971). An overnight culture of the bacteria was diluted in fresh, prewarmed mineral salts + glycerol medium to $OD_{570} = 0.075$ and cultivated at 37 °C in a reciprocal water bath shaker (Gallenkamp IH-350). At $OD_{570} = 0.300$, i.e. in the log phase, at 0 minute the cells were induced with 5×10^{-4} M TMG (methyl-β-D-thiogalactoside, Serva). Shaking was continued and at various intervals after induction D-tryptophan (analytical grade, Reanal) was added to the D-tryptophan-treated cultures at different concentrations. The estimation of β-galactosidase activity in samples of 0.2 or 0.5 ml was the same as described earlier (Schlammadinger, Szabó, 1971). One enzyme unit = 1 μmole ONGP 6(*o*-nitrophenyl-D-galactopyranoside, Fluka) hydrolyzed per 1 ml bacterial suspension* per 10 minutes, at 37 °C.

The experiments were repeated at least three times and the effect of D-tryptophan was tested also on *E. coli* Hfr H Cavalli (*met*$^-$, *rel*$^-$) and ML-3 ($i^+p^+o^+z^+y^-a^+$) strains.

* Prepared under standard conditions.

Fig. 1 shows the inhibitory effect of 10^{-4} M D-tryptophan on induced β-galactosidase synthesis and the reversal of this inhibition by L-tryptophan added in equimolar concentration. Induction started at 0 and 1 minute, in the K12 and Hfr Cavalli culture, respectively. At 15 and 16 minutes one portion of the induced cells of K12 and Hfr Cavalli culture, respectively, were transferred into Erlenmeyer flasks containing D-tryptophan at the final concentration required. Shaking was continued and at 25 and 26 minutes aliquots of these suspensions were added to L-tryptophan at a final concentration of 10^{-4} M. As shown in Fig. 1, 10^{-4} M D-tryptophan almost completely inhibits induced β-galactosidase synthesis, and L-tryptophan given in the same concentration completely reverses the effect of the unnatural compound.

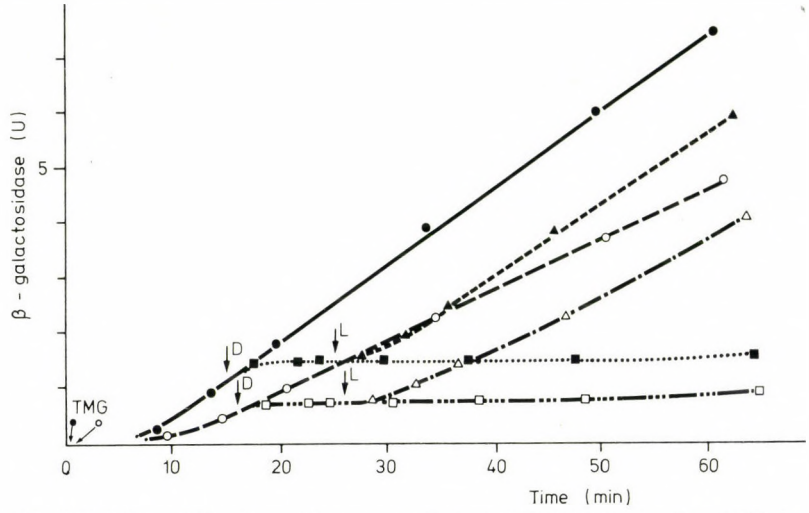

Fig. 1. The inhibition of β-galactosidase synthesis by 10^{-4} M D-tryptophan (added as indicated by arrows marked "D"), and the reversal of this inhibition by 10^{-4} M L-tryptophan (added as indicated by arrows marked "L")

	E. coli K12	*E. coli* Hfr Cavalli
Control	●——●	○------○
10^{-4} M D-tryptophan	■·····■	□—·—□
10^{-4} M L-tryptophan	▲------▲	△—·—△

In other experiments the effect of 10^{-3} M D-tryptophan could be prevented by 2×10^{-4} M L-tryptophan if the latter was applied 5 minutes later. If, however, 10^{-4} M D- and L-tryptophan were added simultaneously, the rate of β-galactosidase synthesis became lower than that of the control culture: it was only about 70% of the control cells in the examined period, i.e. within 35 minutes after the addition of both substances. The curve representing the reversal of inhibition by L-tryptophan resembles the enzyme induction plots. This means that the addition of L-tryptophan to the D-tryptophan-inhibited culture evokes a similar course of enzyme synthesis as the addition of inducer to non-induced cells.

Similar results were obtained when the ML-3 strain was examined.

Surveying the relevant publications we could not find data on D-tryptophan as a potent inhibitor of enzyme synthesis. Its analogue, however, 5-methyltryptophan, has been successfully applied for studying the kinetics of transcription and translation of induced β-galactosidase synthesis in *E. coli* (Kepes, 1969). On the basis of this work we recommend another potent inhibitor of β-galactosidase production, the effect of which can be completely reversed. Enzyme synthesis is arrested by D-tryptophan at a remarkably low concentration and the suspension of this inhibition does not need high doses of L-tryptophan.

In the course of the above experiments we observed the inhibition of induced β-galactosidase synthesis by DL-tryptophan, which was though less expressed. This warns us of the pitfalls of using DL amino acids instead of the natural L-forms in experimentation.

D-tryptophan decreases overall protein synthesis, too, but not in the same manner as with β-galactosidase. Preliminary experiments indicate that this effect is transient and the incorporation of (^{14}C)-L-leucine escapes after a few minutes during the inhibition period, while β-galactosidase synthesis remains completely repressed.

Preliminary experiments with other D-amino acids gave similar results. This field is being further investigated.

We wish to express our thanks to Dr L. Alföldy for the Hfr H Cavalli and to Dr J. Janeček for the ML-3 strain. The skilled technical assistance of Miss Zsuzsa Mórik and Mrs Ibolya Szekeres is appreciated.

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A Simple and Rapid Technique for the Preparation of Viable Leukocytes and Erythrocytes from Human Blood

(Preliminary Communication)

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The separation of different cell components of human blood is very important in haematology and immunology. The recovery of leukocytes by different methods is usually not higher than 30% (Coulson, Chalmers, 1964; Thorsby, 1967). The isolation of leukocytes in good yield and in physiological proportions is a prerequisite for the preparation of different lymphocyte populations. On the other hand blood preparations free from leukocytes and platelets are very desirable in the transfusion practice to avoid immunization with HL–A antigens.

A simple and rapid method was developed for the isolation of leukocytes in relatively high yield and for the production of human blood preparations free from leukocytes and platelets.

ACD solutions was used as anticoagulant. Blood samples not older than 48 hours were applied. The separation of blood cells was performed on microcrystalline cellulose (or DEAE-, or CM-cellulose) swollen in 0.15 M NaCl solution at room temperature for 24 hours. For the chromatographic filtration of 20 ml blood samples 1.4×3.0 cm columns were used. The flow-rate was maintained at 60 ml/hour by a peristaltic pump. During the filtration the white blood cells and platelets were bound to the cellulose while the bulk of the erythrocyte content was eluted. The column was washed with 25 ml 0.15 M NaCl in order to remove the rest of the erythrocytes. The cellulose was removed from the column, suspended in 25 ml 0.15 M NaCl in order to recover the white blood cells bound to the cellulose and allowed to sediment for 15 minutes in a beaker. The supernatant was decanted into a centrifuge tube. This procedure was repeated twice. The combined supernatant fraction was centrifuged ($200 \times g$) and the pellet was suspended in Parker 199 medium containing 10% human AB sera.

The ATP content of erythrocytes was measured after filtration by the method of Bücher (1947), the lactic acid production of the cells was monitored by the method of Barker and Summerson (1941). The number of leukocytes and platelets was counted in samples taken before and after filtration as well as in the suspended pellets.

Leukocytes were found to be adsorbed quantitatively and reversibly to cellulose. The blood samples after filtration did not contain leukocytes or platelets.

The ATP content and lactic acid production of red blood cells in the eluate remained practically unchanged. The viability of lymphocytes recovered from the cellulose was more than 90% as determined by the trypan blue exclusion technique. Table 1 summarizes the results of six typical experiments on the elution of leukocytes from cellulose. 46–60% of the leukocytes were recovered. The proportion of the recovered granulocytes and lymphocytes did not differ markedly from that in normal human blood (1.9–2.3 : 1). The slight red blood cell contamination of the leukocyte suspension could be removed by NH_4Cl /EDTA treatment (Shortman et al., 1972).

Table 1

Recovery of white blood cells on Whatman DE–52 cellulose columns

Blood volume, ml	Number of white blood cells		Yield, %	Column size, cm × cm
	in sample applied	recovered from the column		
80	4.0×10^8	2.4×10^8	60	1.4 × 6
40	2.8×10^8	1.3×10^8	46	1.4 × 4
20	1.0×10^8	0.58×10^8	58	1.4 × 3
20	1.4×10^8	0.65×10^8	46	1.4 × 3
20	1.4×10^8	0.67×10^8	48	1.4 × 3
20	1.4×10^8	0.83×10^8	59	1.4 × 3

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Interaction of Glycolytic Enzymes: Increase of the Apparent Molecular Weight of Aldolase in Rabbit Muscle Extract

(Short Communication)

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The organization of different enzymes into multienzyme complexes has been demonstrated in a number of metabolic systems (cf. Ginsburg, Stadtman, 1970). Such heterologous associations may play a role in both enzyme catalysis and regulation, and hence in the overall functioning of entire pathways. The juxtaposition of functionally relevant enzymes often ensures the "channelling" of metabolite(s), which probably constitutes an important device for intracellular compartmentation.

The detectability of association between enzymes is largely dependent upon the stability of their complex. The so-called "soluble" enzymes, e.g. the glycolytic system, are usually regarded as individual catalytic entities randomly distributed in the compartment that accommodates them. However, under the special conditions of a living cell (very high protein concentration, abundance of membrane surfaces, etc.) there might be interactions even between these enzymes.

With such thoughts in mind we examined whether aldolase, a glycolytic enzyme, interacted with some other component(s) of a concentrated rabbit muscle extract. We chose this enzyme because its association with α-glycerophosphate dehydrogenase (Baranowski, 1939) and with triosephosphate isomerase (Rose et al., 1962) had earlier been suggested. As a method we adopted frontal analysis gel-filtration (Ackers, 1964), which proved to be a suitable technique for the detection of reversible macromolecular associations (Chiancone et al., 1968; Keresztes-Nagy, Orman, 1971).

Rabbit skeletal muscle was minced, extracted with 1 volume of distilled water at 0°C for 50 min, and the fraction between 1.70 M and 2.70 M ammonium sulfate concentration was isolated. The precipitate, referred to in the following as "myogen fraction", was dissolved and dialyzed against 90 mM sucrose solution containing 0.05% sodium azide. The small amount of precipitate formed during dialysis was removed by centrifugation at 9000 × *g* for 40 minutes at 4°C. The clear supernatant was applied, in an ascending manner at 20°C, to a Sephadex G–200 column equilibrated with the same solution. (Azide was required to prevent bacterial growth on the column during the runs.) The volume of the sample

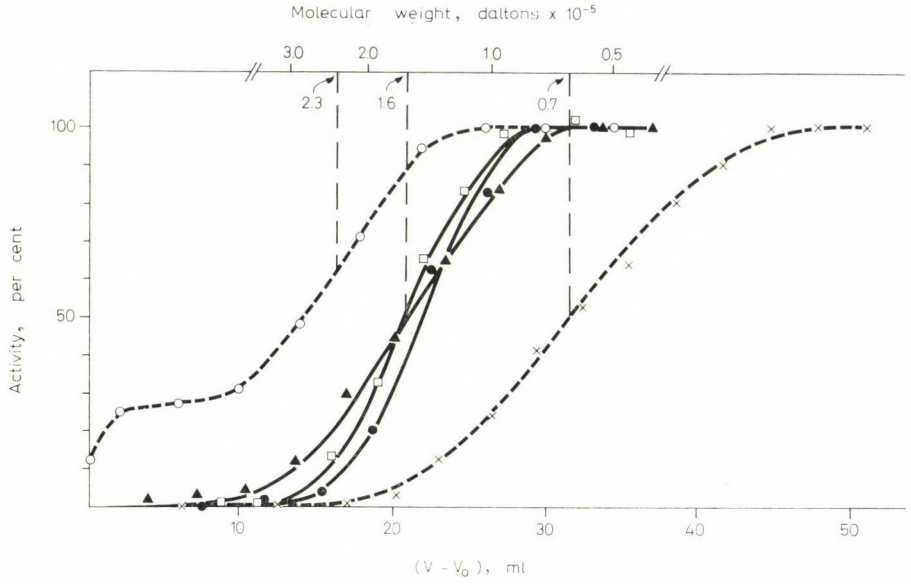

Fig. 1. Elution profiles of aldolase on Sephadex G—200. A 2×37 cm column operated at 20 ± 0.5°C in ascending manner. The column and all samples were in 90 mM sucrose—0.05% sodium azide. 50 ml samples were applied. Flow rate 10 to 12 ml/hour. The column was calibrated with catalase, aldolase, and α-glycerophosphate dehydrogenase. Molecular weights were calculated according to Fischer (1969). The inflection point of sigmoidal elution curves was regarded as the elution volume. The void volume (v_0) was determined with Blue Dextran. Reproducibility of elution volumes ±0.5 ml. Enzyme activities are normalized as per cent of activity in the sample applied to the column. Curves: o—o and x—x aldolase and α -glycerophosphate dehydrogenase activities in the "myogen fraction", total protein concentration 20 mg/ml, containing 120μg/ml aldolase and 180μg/ml GDH resp.; ●—● and ▲—▲ crystalline aldolase, 1.5 and 30 mg/ml respectively; □—□ 2 mg/ml crystalline aldolase, the column equilibrated with 24 mg/ml myoglobin

was large enough to meet the criteria of frontal analysis technique. The elution of aldolase was detected by measuring its activity according to Jagannathan et al. (1956). In control experiments solutions of crystalline rabbit muscle aldolase (Taylor et al., 1948) were run through the column at various concentrations. The elution patterns in a typical set of experiments are shown in Fig. 1. It is seen that crystalline aldolase emerged from the column as expected on the basis of its molecular weight (160 000 daltons, Kawahara, Tanford, 1966; Závodszky, Biszku, 1967), whether applied to the column at low or high concentration. In contrast, aldolase activity in the "myogen fraction" had an elution profile markedly displaced towards higher molecular weights. Furthermore, the elution curve was complex: it reached a low plateau right after the void volume, then it further rose until it attained the level of the extract loaded on the column. It seems that a minor fraction of aldolase is eluted as if it had a very high molecular weight, whereas

its major part has an apparent molecular weight of about 235 000. Such a result is expected if aldolase forms dissociable complexes with some other macromolecule(s) in the extract.

As another control, we equilibrated the column with 24 mg/ml horse myoglobin dissolved in 90 mM sucrose – 0.05% sodium azide, and then we applied a sample containing 2.0 mg/ml crystalline aldolase and 24 mg/ml myoglobin to the column. As shown in Fig. 1, the presence of myoglobin did not affect the apparent molecular weight of aldolase (about 160 000). This experiment may serve as one example that a functionally irrelevant protein does not produce a shift in the elution profile of aldolase.

In another series of experiments a muscle extract was prepared in a different manner. Minced rabbit muscle was extracted with 0.5 volume of 90 mM sucrose – 0.05% sodium azide. The extract was subjected to heat treatment at 40°C for 2 hours. A heavy precipitate formed, which was removed by repeated filtration. (Heat treatment was necessary to remove the readily precipitable components of the extract, which otherwise would have clogged the column.) The clear filtrate was applied to the Sephadex G – 200 column as described above. In this case, too, aldolase emerged from the column with an increased apparent molecular weight (about 260 000), but the elution curve did not exhibit the shoulder observed with the "myogen fraction" prepared by ammonium sulfate fractionation.

It is unclear what is the association partner that causes the shift of aldolase elution profile in the muscle preparations. Glycerophosphate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase, both detected by Warburg's optical test in the direction of NAD reduction, seemed to emerge from the column corresponding to molecular weights not higher than their own, i.e. 78 000 (van Eys et al., 1959) and 145 000 (Előd i, 1958), respectively.

It would be premature to hypothesize about the possible physiological implications of our findings. While preparing this manuscript a paper appeared by Clarke and Masters (1973), who observed a very similar phenomenon with aldolase in rat muscle extract by means of ultracentrifugation. The concurrence of data suggests, at least, that interactions between glycolytic enzymes might exist.

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Electron Microscopic Autoradiographic Localization of Ca in the Muscle Fibril

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The localization of Ca in resting and indirectly stimulated muscles was investigated.

The toe muscles of frog (*Rana esculenta*) were used in the experiments. Nearly 70 per cent of the grains were found above the A-band, 20 per cent above the I-band and 10 per cent above the Z-line.

Electrical stimulation caused a change in the distribution of grains within the A-band but the percentage distribution among the different bands of fibril remained unchanged.

Introduction

Today it is already clear that the inorganic ions play an important part in muscular activity. But there are some problems as to the localization of these inorganic ions and their change during muscular activity. Autoradiographic investigations were performed in this institute with the hope of adding some new points of view to the elucidation of these problems (Ernst, 1966; Kállay, 1972; Kállay, Tigyi-Sebes, 1967, 1967a, 1968, 1969; Tigyi, 1968).

Although Winegrad (1965, 1965a) described in his very important works the localization of muscle Ca, we hoped to obtain newer data by the application of the more physiological circumstances and by the method of electron microscopic autoradiography.

Method

The toe muscles of frogs (*Rana esculenta*) were used. The hind legs of the killed frog deprived of its skin were placed into 100 ml of Ringer solution containing 1000 μCi of ^{45}Ca in a form of $CaCl_2$. The toe muscles of one of the two legs were excited by the stimulation of the nerve while those of the other were not. A 50 Hz electric current was used as a stimulus which was increased in voltage from 0.1 to 30 during the stimulation. The duration of one stimulus was 0.5 sec and the stimulation was repeated at 2 sec intervals.

After one-hour incubation and stimulation the toe muscles were prepared for electron microscopic autoradiography by a method described by us earlier (Kállay, Tigyi-Sebes, 1969). An inactive frog muscle was also prepared as a control.

Electron microscopic autoradiography was carried out according to Caro and Van Tubergen (1962), with the twice diluted Ilford L-4 research emulsion.

Following an exposure time of 40 days in a lightproof box at 2 °C, the autoradiograms were developed and investigated in the same way as described in our previous paper.

Results

More than a thousand grains were counted on 35 electron microscopic autoradiogramsmade of non-stimulated muscles. The number of grains and their percentage distribution above the different parts of muscle fibril can be seen in the first column of Table 1. About 70 per cent of the total grain counts was found above the A-band of muscle fibril, and more than 10 per cent of the remaining 30 per cent of the grains was found right above the Z-line.

Table 1

Grain counts and percentage distributions above the different bands of non-stimulated,stimulated and control muscle fibrils

	Non-stimulated		Stimulated		Control	
	grain number	per cent	grain number	per cent	grain number	per cent
A-band	708	69.3	706	69.4	41	56
I-band	192	18.8	195	19.2	32	44
Z-line	121	11.9	115	11.4	—	—
Total	1021		1016		73	

Similar grain counts and percentage distribution were found on 30 electron microscopic autoradiograms made on stimulated muscle. The 2nd column of Table 1 shows the number of grains and the percentage distribution in this case.

The 3rd column of Table 1 shows the number of grains and the percentage distribution above inactive muscle fibrils. The different grain counts and percentage distributions are due to the different areas of the bands of muscle fibril.

Grain density was calculated from the number of grains, with the areas of different bands taken into account. The grain density was about 3 times greater above the A-band than above the I-band both in the non-stimulated and stimulated muscle (see Table 2). The grain densities above inactive muscle fibrils

Table 2

Grain densities above the different bands of stimulated, non-stimulated and control muscle fibrils

	Grain density $\frac{\text{Grains}}{100\ \mu^2}$	
	above the A-band	above the I-band
Stimulated	30.5 ± 1.2	10.3 ± 0.8
Non-stimulated	26.3 ± 0.8	8.5 ± 0.7
Control	1.8 ± 0.2	1.7 ± 0.3

were equal over the different bands. A greater grain density was found above the stimulated muscle fibril, although the relation of grain density above the A- and I-band remained unchanged. The increase of grain density came to more than 10 per cent.

The good correspondence of the distributions of grains above both the non-stimulated and stimulated muscle was a little surprising. Thus, the distance of more than two hundred grains from the Z-line was measured on the autoradio-

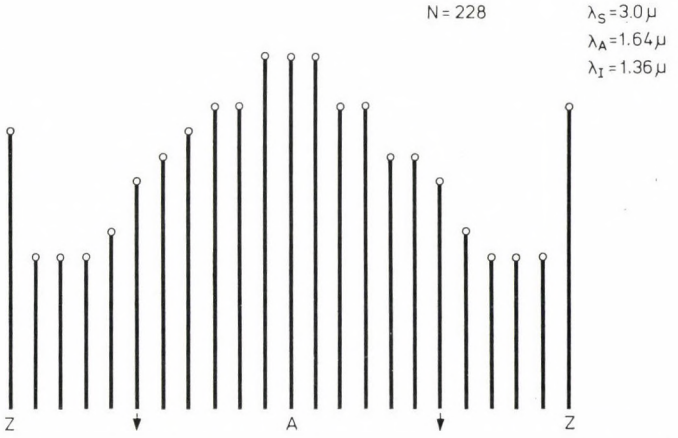

Fig. 1. Line diagram of distribution of grains originated from exchanged ^{45}Ca in resting muscle. The sarcomer length was 3.0 μ. About 230 grains were counted. The arrows show the places of A–I junctions

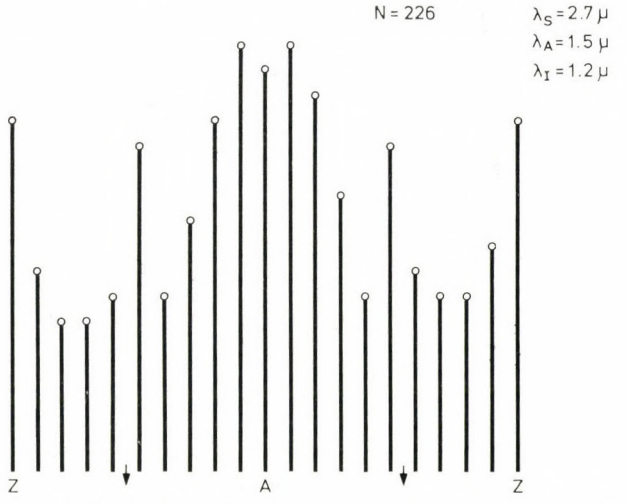

Fig. 2. Line diagram of distribution of grains originated from exchanged ^{45}Ca in stimulated muscel. The sarcomer length was 2.7 μ. About 230 grains were counted. The arrows show the places of A–I junctions

Fig. 3. Electron microscopic autoradiogram of resting muscle after one-hour incubation in Ringer solution containing ^{45}Ca. M × 15000

grams of the non-stimulated and stimulated muscles. The number of grains found on a place of a sarcomer length was presented in a line diagram.

Figure 1 shows the line diagram of non-stimulated muscles. The peaks of grain counts are above the centre of A-band and Z-line, respectively. Figure 2 shows the line diagram of grain distribution of stimulated muscles. In comparison with Fig. 1 the grain counts have two further peaks at the A–I junction within the A-band.

The difference between the distribution of grains can be seen in Figs 3 and 4 as well. Fig. 3 is an electron microscopic autoradiogram of non-stimulated muscle and Fig. 4 is that of a stimulated muscle. Fig. 5 shows the electron microscopic autoradiogram of an inactive muscle.

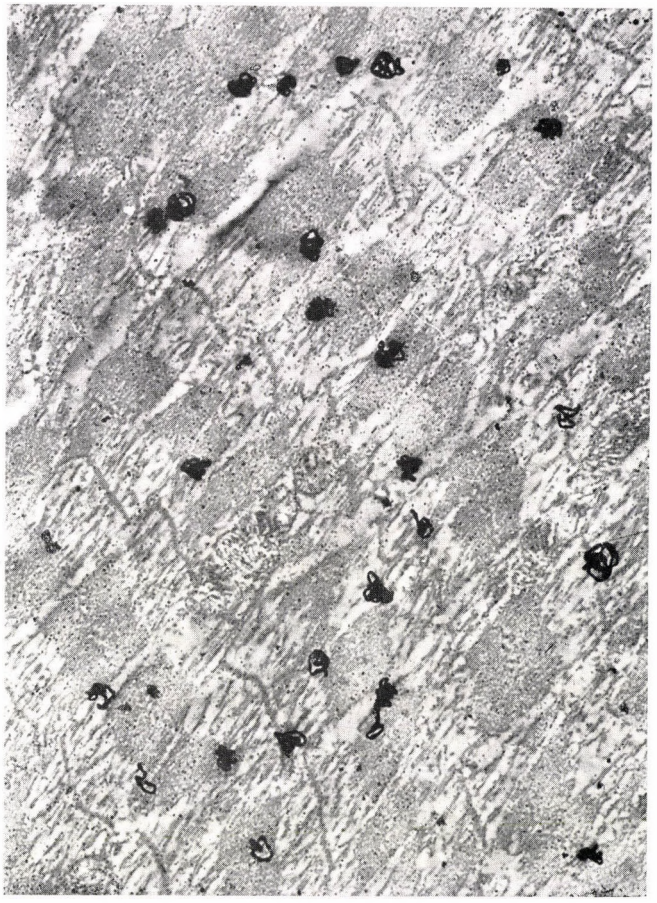

Fig. 4. Electron microscopic autoradiogram of stimulated muscle after one hour incubation and stimulation in Ringer solution containing ^{45}Ca. M $\times$ 15 000

Discussion

By the use of the method of electron microscopic autoradiography it became possible to localize isotopic calcium within the sarcomer of muscle fibril more exactly. Investigations were made to obtain data on the localization of exchanged Ca by incubation of both resting and stimulated muscles. The exchanged Ca in resting muscles was localized in the A-band and the Z-line. A similar localization of Ca was found in stimulated muscles, but there was a change of localization within the A-band. The grain counts had peaks at the A–I junction of muscle fibril.

The peaks of grain densities at A–I junctions were described by Winegrad (1965) as well. He investigated both the longitudinal and transversal distribution

Fig. 5. Electron microscopic autoradiogram of inactive muscle (control). M × 15 000

of exchanged Ca by light microscopic autoradiography. He noticed on the longitudinal distribution of exchanged Ca that most of it was localized in the 0.2 to 0.3 μ centre of the I-band and in the part of the A-band where the thick and thin contractile filaments overlap. He did not use any stimulus in his experiments.

The peaks of the grain counts at the A–I junctions (the so-called overlapping zone) were found in our experiments only in the case when the muscles were stimulated. So, without any doubt as to the role of Ca also in the structure of muscle fibril, we attribute this change in the Ca-distribution to muscular activity.

The authors thank Professor E. Ernst and Professor J. Tigyi for their encouragement during this work.

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Effect of Denaturation on Muscle Proteins as Studied by Epr

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The McConnell spin label technique with SH-directed label was employed on surviving frog fibre bundles and on glycerinated rabbit psoas fibres in order to study the conformational changes in muscle proteins during urea and guanidine hydrochloride induced denaturation. The spin labeled muscle proteins showed a complex epr spectrum, which was due to the restricted motion of label. The correlation time was 1.43 nsec for weakly immobilized spin label and about 15 nsec for the strongly immobilized spin label in the case of surviving fibre bundles from frog.

It has been concluded that the environment of spin label changes during denaturation evoked by urea and guanidine hydrochloride. The strongly immobilized signal disappears, and a significant increase can be observed in the average mobility of spin label. The conformational changes were found to be reversible at every urea concentration applied in the experiments.

Introduction

The spin-label method developed by McConnell (McConnell, McFarland, 1970; Hamilton, McConnell, 1968) is very sensitive and reports changes in conformation and is, therefore, well suited to study biological problems. The epr spectra give information on the rotational freedom of spin labels, and any change of the conformation of the proteins which affects the rotational freedom of label can be detected.

The contractile system of muscle has been widely investigated in order to obtain information concerning the molecular events which actually exist during contraction or other artificially induced changes. The structural effects, which control the mechanical activity of the striated muscle, the shortening and tension-development are still unknown. Cooke and Morales (1969), Quinlivan et al. (1969) and Tonomura and his co-workers (1969) carried out experiments on glycerinated muscle fibres and model systems using the McConnell spin-label technique in order to study the conformational changes of muscle proteins as the fibres contract or the myosin is modified. We have extended the spin-label experiments to surviving muscle fibres to avoid the effect of glycerination and studied the effect of denaturation.

Abbreviations used: epr, electron paramagnetic resonance; IAA, 4-(2-iodoacetamido)-2.2.6,6-tetramethyl-piperidinooxyl spin label; IC, isocyanate spin label.

Materials and methods

Freshly prepared fibre bundles of m. sartorius from frog (*Rana esculenta*) were immersed and kept in Ringer solution (115.5 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$ and 2.4 mM $NaHCO_3$ in 1000 ml bidistilled water) in a refrigerator at 4 °C for a few hours. Thereafter the fibre bundles were labelled in Ringer solution with 4-(2-iodoacetamido)-2,2,6,6-tetramethyl-piperidinooxyl spin label (purchased from Synvar Associates) for 12–24 hours. The concentration of the spin label in solution was $5 \cdot 10^{-4} - 5 \cdot 10^{-5}$ M. During incubation the preparations were stored in a refrigerator at 4 °C. After labeling, the physiological state of the fibre bundles was controlled by recording the action potential evoked by electric stimulation. More than 80 per cent of the fibres prepared was yet excitable, that is, the applied concentration required for measurements had almost no effect on the electric activity of the muscle preparations (see Hubbell, McConnell, 1968).

In order to compare the experiments performed on surviving fibre bundles, glycerinated fibre bundles were prepared from m. psoas of rabbit according to the method described by Huxley (1963). The fibre bundles were glycerinated for 3 weeks. After washing the fibre in 0.1 M KCl, 0.001 M $MgCl_2$ and 0.07 M phosphate buffer to remove glycerol, we kept the preparations in the same solution containing the IAA label in $5 \cdot 10^{-5} - 5 \cdot 10^{-4}$ molar concentration in a refrigerator at 4 °C for 25 hours. The fibre bundles were repeatedly washed in salt solution before epr spectra were recorded.

The electron paramagnetic resonance spectra were taken at room temperature using a Carl Zeiss Model ER 9 spectrometer operating at 9650 Mc/s. All spectra of fibre bundles were recorded at the same power setting, the modulation frequency was 100 kc/s and the amplitude was chosen to be 1.84 and 0.92 G, time constant 4.4 s, the run was 14 min. In most cases the higher modulation amplitude was necessary because of the low concentration of spin label. Field calibration was done by using peroxylamine disulphonate ion radicals in low concentration.

The muscle preparations were put into the quartz liquid flat cell supplied by Zeiss or small glass capillary tubes about 2 mm in diameter.

In order to adjust the length of the fibre bundles another type of cell was developed from teflon, in which the fibre, bound by threads at both ends, could be stretched to the desired length. Before spectra were taken the fibre bundles were washed in Ringer solution at room temperature for 45–60 min, in order to remove unbound labels. The fibre bundles weighed about 20 mg and their long axis was perpendicular to the applied magnetic field.

Results and discussion

Labeling of the striated muscle fibre bundles from frog and glycerinated fibre bundles from rabbit resulted in a complex epr-spectrum. The spectra obtained can be assumed to arise from spin labels which are covalently bound to the muscle proteins. The analysis of the spectrum indicates that the spectrum consists of

two species with superimposed spectra. Fig. 1 and Fig. 2 show the typical epr-spectrum of IAA labelled frog striated muscle and glycerinated fibre bundles from rabbit psoas. The spectrum of the nitroxide type label is similar to that first published by Stone et al. (1965) on BSA labelled with IC. The solid arrows in Fig. 1 show the spectrum due to strongly immobilized spin labels, while the three broad peaks represent the weakly immobilized spin labels with greater degree of rotational freedom (dotted arrows).

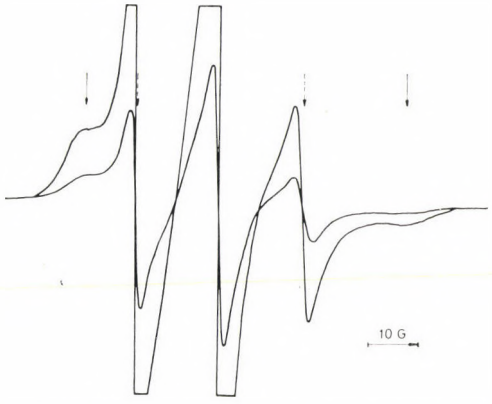

Fig. 1. Spectrum of IAA labelled fibre bundle of frog sartorius. The solid arrows show the peaks arising from strongly immobilized spin label. The magnetic field increases to the right (horizontal arrow). The second run was made by 5 × gain

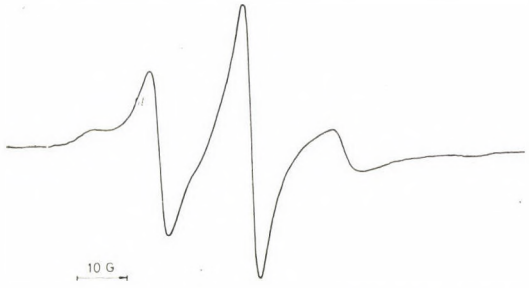

Fig. 2. Epr spectrum of IAA labeled fibre bundle of glycerinated rabbit psoas fibres. The magnetic field increases to the right (horizontal arrow)

As regards the local conformational changes, the rotational correlation time of a protein bound spin label can yield some information. We have used the practical equation derived by Keith et al. (1970), or given by Ignatyeva and Ruuge (1972) and Kuznetsov et al. (1971) to calculate the correlation time:

$$\tau = 8.4 \cdot 10^{-10} \Delta H_{+1} \left(\sqrt{\frac{I_{+1}}{I_{-1}}} - 1 \right) \text{sec}$$

where I_{+1}, I_0, I_{-1} are the heights of the low-, mid- and highfield lines on the first derivative spectrum, and ΔH_{+1} is the width of the low- field component, measured in gauss.

In order to obtain informations on the extent of asymmetry of the spin label environment we have calculated the asymmetric parameter published by Wasserman et al. (1971) by the equation

$$\varepsilon = T_2^{-1}(+1) - T_2^{-1}(0)/T_2^{-1}(-1) - T_0^{-1}(0),$$

replacing the ratio

$$T_2^{-1}(+1)/T^{-1}(0) \quad \text{by} \quad (I_0/I_{+1})^{\frac{1}{2}}$$

for convenience of measurement.

A detailed analysis of the spectra indicates that, on the average, the correlation time of the protein bound spin label is $\tau = 1.43 \cdot 10^{-9}$ sec while $\varepsilon = 0.24$. When the fibre bundles were reacted with IAA in stretched state (about 20 per cent over the labeling length) we have observed a slight decrease of the asymmetric parameter, $\varepsilon = 0.22$. The difference obtained in the experiments is significant. The significance was calculated by the *t*-test at P = 0.01 level from 46 and 17 different spectra, respectively. It was necessary to use statistical calculation because we have experienced small deviations of about 5 per cent in the spectrum parameter within the same experiment.

By an extrapolation method, which is not as correct as that published by McCalley et al. (1972) and Shimshick and McConnell (1972), we have estimated the rotational correlation time of the strongly immobilized spin label. It is about 15 nsec, measured on fibre bundles from frog sartorius.

We have investigated the urea – and guanidine-hydrochloride – induced denaturation of muscle fibres. The spectral lines decrease in width and increase in height with increasing urea or guanidine-HCl concentration. Fig. 3 shows the changes in spectral lines evoked by 6 M urea.

According to Berliner (1972) the changes in spectral lines signify an increase in the average mobility of the spin label, the disruption of the secondary and tertiary structures and a partial unfolding or loosening of the polypeptide chain surrounding the label. The ratio $\sqrt{I_0/I_{-1}}$ plotted against urea concentration is shown in Fig. 4. It should be noted that the strongly immobilized label spectrum, which indicates that the spin label is probably held rigid at the sulfhydryl groups of the myosin molecules, completely disappears during denaturation. The process is reversible to the 0 M urea spectrum at every urea concentration used in the experiments after washing the preparations in Ringer solution of normal composition for 15 min. This phenomenon can be repeated many times. However, it is interesting to note that the reversibility of the changes is not exactly complete in samples denaturated in guanidine-hydrochloride.

We found the weakly immobilized label spectrum to become a little more mobile. This observation is in accordance with the experiments published by

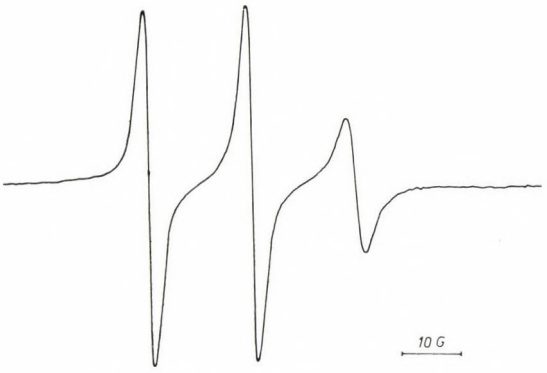

Fig. 3. Epr spectrum of IAA labelled fibre bundle of frog sartorius denaturated in 6 M urea

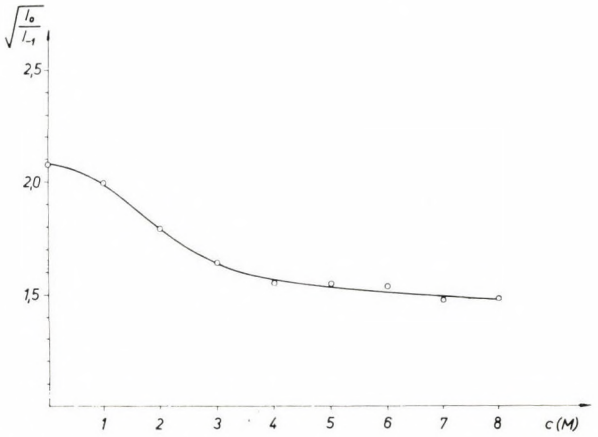

Fig. 4. Concentration dependence of the $(I_0/I_{-1})^{\frac{1}{2}}$ ratio for different preparations. The values are the mean of several determinations

Perrin and Monod (1963) on other proteins. The experiments carried out on proteins extracted from rabbit muscle will be published in a following paper.

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Further Data on the Radiation Injury of MS2 Phages*

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The authors compare their radiobiological results on MS2 phages with the results to be found in the literature. They show quantitatively that in the model previously constructed by them to interpret the injury of phage-RNA the reversible injuries mean dimerization, the irreversible ones uracil-hydration. By the aid of the radiation kinetic model they determine the ratio of reversible and irreversible photoproducts for extremely high doses too. They show that, in case of sufficiently high doses, the quantity of irreversible photoproducts is dominant.

Introduction

In our previous works (Rontó, Tarján, 1972; Rontó et al., 1972) we constructed a radiation kinetic model in connection with the UV-radiation injury of the T7 phage, a bacteriophage containing a double stranded DNA. Based on results obtained from the mathematical formulation of the model we could follow the process of injury from the primary physical event, the absorption of the photon to the development of biological injury, the inactivation of plaque forming activity. As a consequence of this we had shown in an indirect way that in the case of T7 phage the UV injury was manifested at molecular level in the injury of transcription.

It is well known that the single-stranded RNA of phages undergoes partly translation, partly transcription in the course of phage development. Thus the explanation of the radiation injury mechanism of these phages might reveal the role of translation and RNA-reproduction in the development of biological injuries. Among these processes, as a first step the explanation of the nature of the structural injury seems to be indicated. Previously (Karczag et al., 1972a; Karczag et al., 1972b; Rontó et al., 1972) we gave account of the exact determination of the dose-effect curve of the MS2 phage for UV-inactivation and of the radiation kinetic model for the interpretation of the curve. In the present paper we want to compare our results with those to be found in the literature on the UV radiation injury of RNA in order to obtain further information on the nature of our assumed model's injuries, partly reversible and partly irreversible to UV light.

* Based on a paper read at the VIIth Congress of the Hungarian Biophysical Society

The nature of injuries in RNA

In the last decade it became clear that U-U dimer and U-hydrate are produced in the oligonucleotides containing U upon the effect of UV light (Wierzchowski, Shugar, 1962; Brown et al. 1966). Pearson, Johns et al. carried out very extensive experimental studies on longer polynucleotide chains, namely on poly-U and poly-(A+U) complexes to find the U photoproducts (Johns et al., 1964; Johns et al., 1966; Pearson, Johns, 1966; Pearson, et al., 1966); they also published quantitative data on them.

The cited authors stated that the secondary structure considerably influenced the formation of photoproducts. Since, according to the data of Cory et al. (1970) and Jeppesen et al. (1970), the phage RNA contains several loops stabilized by H-bands and within the loops the RNA has a structure similar to the double chain, we want to compare our data mainly with those of Pearson and Johns (1966) on poly-(A+U) complexes. The hydration (h) and dimerization (k) cross-section values interesting for comparison are summarized in Table 1. The letters

Table 1

	Hydration		Dimerization	
	h		k	
	$\frac{cm^2}{\mu E}$	$\frac{\text{base}}{\text{abs. photon}}$	$\frac{cm^2}{\mu E}$	$\frac{\text{base}}{\text{abs. photon}}$
poly-(A+U)*	0.008	1.6×10^{-4}	0.008	1.6×10^{-4}
poly-U*	0.085	1.7×10^{-3}	0.038	7.6×10^{-4}
MS2		$\delta=1.7\times10^{-4}$		$\alpha=1.3\times10^{-4}$

* Data of Pearson and Johns (1966); applied wavelength: 280 nm.

h and k give the probability for 6×10^{17} photons of 280 nm wavelength falling on 1 cm^2 to cause hydration or dimerization on one single U base. As the α and δ probabilities used by us for reversible and irreversible injuries differ from the above probabilities by the fact that they concern one absorbed photon, the values h and k were calculated according to this. In Table 1 we listed the values for one absorbed photon as well. Our own α and δ values are also given in the Table.

From the Table one can see that our α and δ values show good agreement with the k and h values obtained for poly-(A+U), which can be less said for the data on poly-U. This shows that concerning secondary structure the MS2-RNA is more similar to poly-(A+U).

The nature of photoproducts in case of extremely high doses

In connection with the radiochemistry of the UV injury of R17 phage Cerutti et al. (1969) published results. The comparison of these with our data on MS2 phages is especially interesting, because this phage is very similar to MS2.

In its RNA approximately the same number of nucleotide bases are to be found as in the MS2 RNA. The base composition of the nucleic acid is also similar in case of both phages: the C and U give 25 – 25 per cent of the whole number of bases.

Cerutti et al. (1969) studied the photoproducts with a method much less sensitive than the biological one, which made it necessary for them to irradiate the phages with doses much higher than those applied by us causing biological effect. The incident intensity (Johns et al., 1964) was $0.9-3.4\ \mu E/cm^2$ which calculated for the number of photons absorbed by one phage corresponds to $3\times10^5 - 1.1\times10^6$ $\frac{\text{absorbed photons}}{\text{phage}}$. This value is by one–two orders of magnitude greater than our maximal dose: 5×10^4 $\frac{\text{absorbed photons}}{\text{phage}}$. Thus the experimental results of Cerutti et al. can be compared to our data only with the aid of our radiation kinetic model.

In the course of comparing the radiation injuries to the two phages we are connected to the following two problems:

1. Cerutti et al. (1969) stated that about 10 per cent of the R17 uracil would be in a hydrated state upon the effect of $2.2\ \mu E/cm^2$ incident dose. Based on our model we seek an answer to the number of expectable irreversible injuries in case of such an extreme dose.

2. According to the studies of the authors cited there were no dimers present in a measurable quantity in the phage RNA under their experimental conditions. In connection with this we are seeking an answer to the ratio of reversible and irreversible injuries in one phage in case of much higher doses.

By the aid of our model both questions can be answered, because the probability of an arbitrary number of reversible and irreversible injuries in a given population can be calculated for any arbitrary dose.

Some simplifications were made in the calculations: a) We considered the number of injurable places constant, since according to the experience of Cerutti et al. only 10 per cent of the uracils suffered irreversible injury in case of the doses applied by them. b) We considered the values of the α, γ, δ transition probabilities also constant.

To carry out the calculation we made a program for an IBM computer. Our results are shown in Figs 1a – d. These show the probabilities of a number of 0–k reversible and irreversible injuries at different absorbed doses $\left(6\times10^3,\right.$ 1.2×10^4, 3.6×10^4 and $\left.5\times10^4\ \frac{\text{absorbed photons}}{\text{phage}}\right)$. From Figs 1a and 1b one can see that the distribution of reversible and irreversible injuries is very similar in case of such relatively low doses. 0 or 1 hi ts are to be found with the highest probability in the irradiated population. In Fig. 1c taken with a dose higher than the previous one $\left(3.6\times10^4\ \frac{\text{absorbed photons}}{\text{phage}}\right)$ the curve giving the distribution of irreversible injuries shifts to the right, to the higher number of injuries, with a maximum at 4 injuries. This shift is even more pronounced in case of a

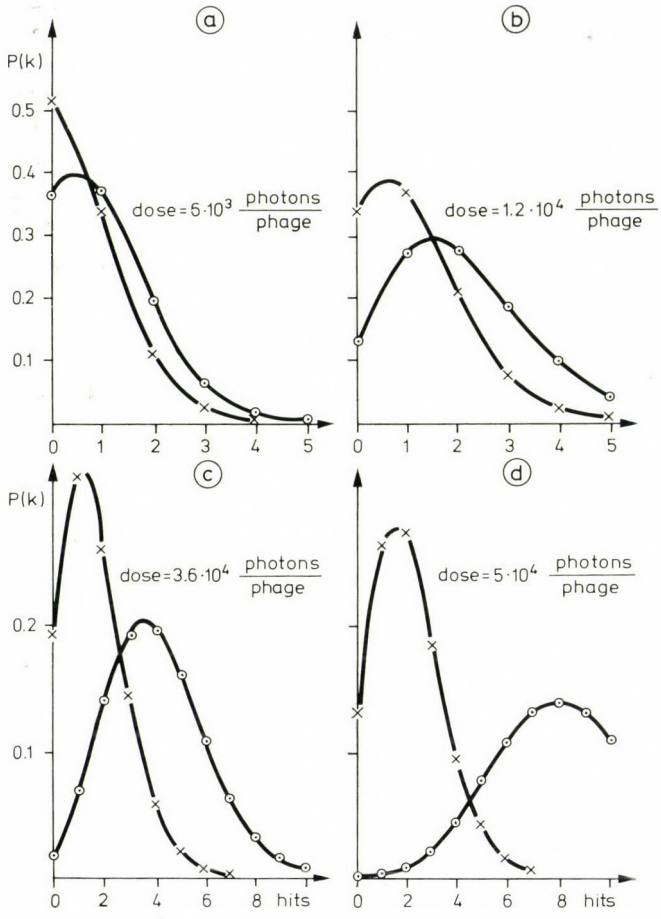

Fig. 1. The probabilities of reversible (×) and irreversible (○) hits in one phage based on our radiation kinetic model. Doses: a) 6×10^3, b) 1.2×10^4, c) 3.6×10^4, d) 5×10^4 $\frac{\text{absorbed photons.}}{\text{phage}}$

higher dose $\left(5\times10^4 \frac{\text{photons}}{\text{phage}}\right)$ (Fig. 1d) . While the maximum of the distribution of reversible injuries is at the double injury this maximum is at 8 in case of irreversible injuries.

Though it is not plotted in the Figure, this maximum is shifted even more to the right at a dose of 5×10^5 $\frac{\text{absorbed photons}}{\text{phage}}$. The most probably expectable number of reversible injuries is 2, while the most probable number of irreversible injuries in one single phage is $83-86$.

We determined the above shown distributions for even higher doses. The results are summarized in Fig. 2 where the maxima of the distributions of rever-

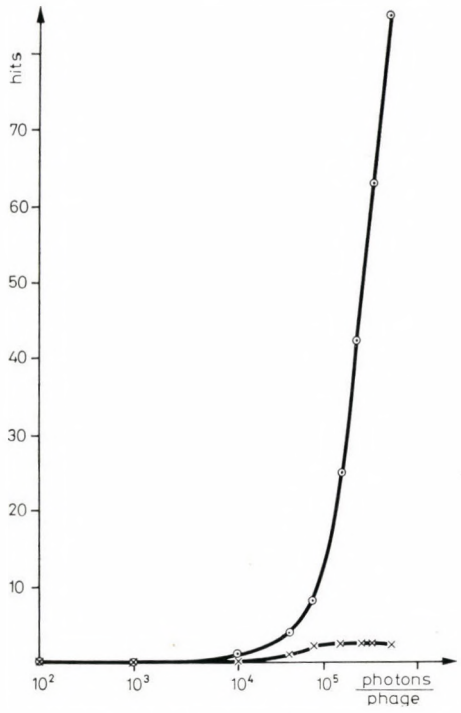

Fig. 2. The variation of the most probable number of reversible (×) and irreversible (○) hits in dependence of the number of absorbed photons per phage

sible and irreversible hits are plotted against the number of absorbed photons per phage.

It can be seen from Fig. 2 that at such low doses $\left(10^2-10^4 \frac{\text{absorbed photons}}{\text{phage}}\right)$ causing inactivation well measurable with biological methods the probability of reversible and irreversible injuries are nearly equal and usually $1-2$ such injuries are expectable in one phage. With the increase of dose the irreversible injuries become more and more dominant because, owing to a dynamical equilibrium, the number of injuries revertable by light remains constant (the number of reverted injuries is equal to the number of new injuries). Thus even at very high doses $\left(5\times10^5 \frac{\text{photons}}{\text{phage}}\right)$ most probably only 2 reversible injuries occur in one phage while 85 irreversible injuries are expectable.

Both of our questions can be answered on the basis of Fig. 2 or what was said above.

Summing up our results we can state that though our model was mainly constructed to interpret biological inactivation it also seems to be applicable for the comparison of the biological effect and the radiochemical experience.

This comparison has also proved that in the phage-RNA the *reversible injuries* must be *considered C-C, U-U and U-C dimers and the irreversible injuries hydrated uracils.*

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Book Review

Biophysikalische Aspekte des Alterns multizellulärer Systeme. W. Beier, K. H. Brehme, D. Wiegel (Fortschritte der experimentellen und theoretischen Biophysik, Bd. 16, Edited by W. Beier, Leipzig). VEB Georg Thieme, Leipzig, 1973

The booklet presents a mathematical model for the description of the ageing phenomena of multicellular organisms. It is designed to be the basis of treatment of experimental and clinical results of the authors, participants in a complex research program of gerontology.

The work consisting of 69 pages is divided into three main chapters. After a short introduction the second chapter describes the mathematical model proper. A complex function of state, the vitality, was chosen by the authors as its base, whose decrease in time is considered characteristic of the process of ageing. "Vitality" actually means here the difference of two functions of state: the processes of development as well as the deviation from it, the "development of error", so the point under discussion is a two-factor model. In the following this chapter deals with the deduction from the basic function of a series of quantities which can be used for the characterization of ageing and with the presentation of its usefulness for different types of development, respectively. At the end of the chapter the authors examine how suitable the applied model is for reflecting the ten most important empirical facts enumerated by Shock which are usually characteristic of the ageing organisms, and they state that the model is suitable for this purpose.

The third chapter presents a survey of the literature of the cellular and molecular aspects of the ageing of multicellular organisms and the possibility of their mathematical modelling.

The booklet is supplemented by a list of references selected by emphasizing the essentials and consisting of 127 items. The work well exemplifies how we can approach the everyday questions of practical biology and medicine from the basis of theoretical biology.

P. JUHÁSZ

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