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Effect of Thiol Blocking and Substrate Binding on the Conformation and Conformational Stability of Rabbit Muscle Aldolase

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(Received November 4, 1971)

Dedicated to Professor A. E. Braunstein in commemoration of his 70th birthday

1. Blocking of two thiol groups per subunit of aldolase with PMB renders the structure of the enzyme less compact, as shown by ORD and viscosity studies Concomitantly the conformational stability of the enzyme decreases as concluded from heat inactivation and hydrogen-deuterium exchange experiments. In spite of the conformational changes the substrate- and the specific P_i-binding sites of aldolase are not damaged. This fact would account for the full activity of aldolase which contains two mercaptide bonds per subunit.

2. Binding of specific ligands, substrate or P_i, to either native or mercaptidated aldolase greatly increased the conformational stability of the proteins without detectable changes in the overall conformation.

Introduction

The thiol groups of aldolase were found to exhibit differential reactivity towards PMB, dithio-bis nitrobenzoate and bromacetate (Swenson, Boyer, 1957; Szabolcsi, Biszku, 1961; Eagles et al., 1969; Szajáni et al., 1969). Our previous investigations (Szabolcsi, Biszku, 1961; Szabolcsi et al., 1964) have shown that blocking of the most reactive SH group** with PMB is sufficient to alter the conformation of the enzyme, this change being reflected in an enhanced tryptic susceptibility. Digestion of the enzyme in which an additional sluggish-reacting SH group is blocked with PMB proceeds even faster, but even this derivative — denoted as aldolase-$(SHg)_2$ — is still fully active.

Abbreviations: PMB, p-mercury-benzoate; FDP, fructose-1,6-diphosphate; aldolase-$(SHg)_2$, aldolase containing 2 mercaptide bonds per subunit.

Enzyme, Fructose-1,6-diphosphate: D-glyceraldehyde-3-phosphate lyase (EC 4.1.2.13).

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** In earlier studies the number of SH groups has been expressed per mole protein. Since several investigations suggest an equal distribution of thiol groups in the four polypeptide chains of aldolase (Sajgó, 1969; Anderson et al., 1969; Szajáni et al., 1970), we have alculated the number of reacted thiol groups per subunit.

In the present work we followed two lines. We studied 1) the conformational changes induced by the blocking of two thiol groups per subunit of aldolase, and 2) the differences in the conformational stability of native aldolase and aldolase-$(SHg)_2$.* Furthermore, we studied the effect of specific ligands, substrate and P_i, on the conformation and conformational stability of native aldolase and the mercury derivative.

The conformational changes were investigated by ORD and viscosity measurements. The conformational stability was studied by measuring the rate of heat inactivation at pH 7.5, at 58° and hydrogen-deuterium exchange rates of the peptide hydrogens at pD 7.5 at 20°. The kinetics of hydrogen-deuterium exchange of peptide hydrogens were assumed to reflect the rapid conformational transitions which occur within the protein molecule (cf. Hvidt, Nielsen, 1966). The peptide hydrogens of proteins are usually classified into the following groups: a) rapidly exchanging hydrogens, b) those exchanged at a measurable rate, c) hydrogens hard to exchange. The rapidly exchanging hydrogens most probably represent peptide hydrogens fully exposed to the solvent. Slowly exchanging hydrogens (category b and c) probably belong to buried segments of the molecule, inaccessible to the solvent. Exchange from these "buried" segments can occur if they become exposed in conformational intermediates. Investigations of the pD and temperature dependence of hydrogen-deuterium exchange in chymotrypsin (Rosenberg, Chakravarti, 1968; Rosenberg, Enberg, 1969) and in aldolase (Závodszky, Abaturov, 1971) support the view that at least two pathways exist for the exchange of retarded peptide hydrogens: (i) the reversible cooperative thermal unfolding of buried segments, which requires high activation energy and (ii) non-cooperative motion of segments near the surface region of the protein molecule. Transition (ii) requires lower activation energy. Under the experimental conditions applied in the present studies, 20° and pD 7.5, the segmental non-cooperative motions are predominant, therefore the rate of hydrogen-deuterium exchange would reflect changes in rate of segmental motions in any part of the molecule, i.e. the motility of the protein.

Materials and methods

Aldolase was prepared and recrystallized three times from rabbit skeletal muscle according to Taylor et al. (1948). Enzyme solutions were gel-filtered on a Sephadex G-50 column equilibrated with 0.1 M Tris buffer, pH 7.5, in the cold. Enzyme concentration was determined spectrophotometrically, by using the extinction coefficient: $E_{280}^{0.1\%} = 0.74$ (Biszku et al., 1964). All calculations were based on a molecular weight of 160 000 (Kawahara, Tanford, 1966; Závodszky, Biszku, 1967). Specific activity of the preparations varied between 130 and 160, as determined by the dinitrophenyl hydrazine test (cf. Enzyme assay).

FDP was purchased from different companies (Reanal, Sigma). All preparations contained some contaminations which markedly increased the rate of heat

* As to the interpretation of conformational stability cf. Tanford (1968).

inactivation of aldolase. Therefore FDP was purified according to Mandl and Neuberg (1957).

PMB (Calbiochem) was purified as described by Boyer (1954).

Tris(hydroxymethyl)-aminomethane was recrystallized twice from 70% ethanol. Phosphate buffer was prepared from Na salts. All other chemicals were commercial preparations of reagent grade.

Enzyme assay was performed according to Swenson and Boyer (1957) by the dinitrophenyl hydrazine test at 30°, pH 7.5 in 0.1 M Tris buffer as described previously (Szajáni et al., 1970). Specific activity was calculated according to Swenson and Boyer (1957) from the relationship S.A. $= \Delta E_{520}^{10'}$ divided by the absorption of the enzyme at 280 nm in the test system.

PMB treatment. 1–10 mg/ml aldolase solutions gel-filtered in 0.1 M Tris buffer, pH 7.5, were incubated with 8 moles of PMB per mole enzyme at 4° for 40 minutes. Mercaptide formation was determined by Boyer's spectrophotometric test (Boyer, 1954). The number of mercaptide bonds formed is expressed per subunit (mol. weight 40 000). Specific activity of aldolase-$(SHg)_2$ equalled that of the native enzyme.

Heat inactivation was carried out as described previously (Szajáni et al., 1970) in 0.1 M Tris buffer, pH 7.5, at 58°. Concentrated solutions of native and modified aldolases were diluted to 1 to 10 mg/ml protein concentration with 0.1 M Tris buffer, pH 7.5, at 58°. The diluting buffer also contained 0 to 0.2 M sodium phosphate buffer, pH 7.5, or 0 to 1.3 mM FDP. If FDP was present in the system, enzyme and substrate were preincubated at room temperature until equilibrium was reached. The progress of denaturation was monitored by measuring residual enzymic activity. Inactivation followed first order kinetics up to about 95% loss of activity.

ORD measurements were carried out in the wavelength range 300 to 360 nm in a 1 cm cell, with about 0.5% protein solutions. The accuracy of readings was $\pm 0.003°$. The contribution of FDP to the rotation was taken into account. The values of a_0 and b_0 were calculated from the Moffit–Yang equation (1956). The mean residue weight (MRW) was taken to be 110 from the amino acid composition (Lai, 1968) and $\lambda_0 = 212$.

The viscosity of 0.1 to 1.0% protein solutions was determined in Oswald viscosimeters at 20° (outflow with distilled water 160 sec.). As solvent 0.1 M Tris buffer, pH 7.5, was used.

Hydrogen-deuterium exchange was examined by infrared spectrometry as described earlier (Závodszky et al., 1966) at pD 7.5 and 20°. The disappearance of the absorption at 1550 cm^{-1} (amide II band) was followed from 2–3 minutes after dissolving the lyophilized protein in D_2O. The absorption at 1650 cm^{-1} (amide I band) was taken as an intrinsic reference, since it is proportional to protein concentration and is not affected by hydrogen-deuterium exchange. Exchange was calculated from the amide II/amide I absorption ratio. The zero time value (0.58) was measured in lyophilized thin layers soaked with paraffin. A similar value, 0.6, is obtained from the extinction coefficients of =CO and –NH-groups (Di Sabato, Ottesen, 1965).

Before lyophilization the enzyme solutions were gel-filtered in 0.1 M Tris buffer. If the effect of P_i or FDP on the exchange was examined, phosphate buffer and substrate solution were separately lyophilized and the lyophilized protein samples were dissolved in the D_2O solutions of P_i and FDP, respectively. The pD value of the samples was controlled with a Radelkisz OP-205 precision pH-meter. The actual pD value was calculated according to Glaroe and Long (1960): pD = pH + 0.4. The experiments were carried out in a UR-10 Zeiss GDR double beam recording infrared spectrophotometer.

Lyophilization did not affect the specific activity of either native or modified aldolase. Enzymic activity of the samples was tested at the beginning and at the end of each experiment. Only those experiments were accepted in which the specific activity did not decrease by more than 20%.

Results and discussion

Effect of thiol-blocking on the conformation and conformational stability of aldolase

Blocking of two thiol groups per subunit of aldolase provokes marked changes in the conformation of the enzyme (Table 1). Both the ORD parameters and the intrinsic viscosity indicate that the structure of the fully active aldolase-$(SHg)_2$ is less compact than that of the native enzyme.

Table 1

Some physical-chemical parameters of native and modified aldolases

Specific activity of both species of aldolase was 140

Enzyme	Moffitt parameters		Intrinsic viscosity [η], dl/g	Rate constant of inactivation, pH 7.5 and 58° k × 10, (min^{-1})	Exchangeable peptide hydrogens at pD 7.5 and 20°			
					class I		class IV	
	$-a_0$	$-b_0$			$t_{1/2}$ min	% *	$t_{1/2}$ hour	%
Native aldolase	58	160	0.034	1.5	<1	50	53.5	37
Aldolase-$(SHg)_2$	65	140	0.039	2.75	<1	56	41.3	30

* Zero time value = 0.58 was taken as 100% (cf. Methods)

The structural changes are also reflected in a decreased conformational stability. We compared the kinetics of heat inactivation of the two enzyme species at 58° and pH 7.5 and the kinetics of the hydrogen-deuterium exchange of the peptide hydrogens at 20° and pD 7.5. The rate constant of heat inactivation of the mercury derivative is increased by a factor of about 2 (Table 1). The time

course of hydrogen-deuterium exchange is presented in Fig. 1, curves 1 and 2. The overall process of exchange which represents the sum of a number of first order reactions has been graphically resolved into four first order reactions (cf. Hvidt, Nielsen, 1966). Class I represents peptide hydrogens with an average half exchange time less than 1 min, classes II and III the hydrogens exchanging at

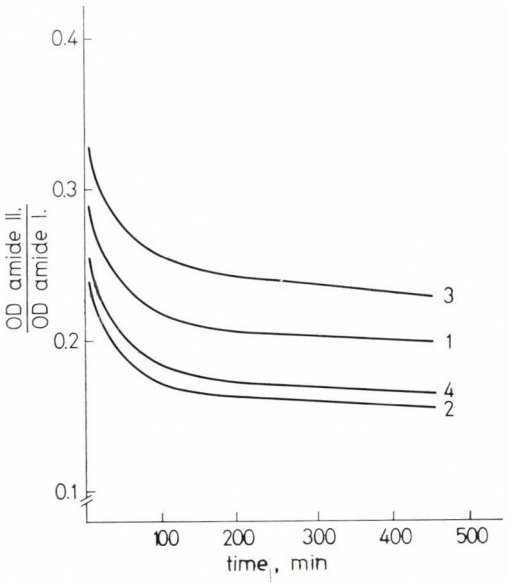

Fig. 1. Time course of hydrogen-deuterium exchange at pD 7.5 and 20°. Curve 1: native aldolase in 0.1 M Tris buffer; Curve 2: aldolase-$(SHg)_2$ in 0.1 M Tris buffer; Curve 3: as curve 1 plus 50 mM P_i or 70 mM FDP; Curve 4: as curve 2 plus 50 mM P_i or 70 mM FDP Zero time value = 0.58 (cf. Methods). The measurements started 3 minutes after dissolving the ingredients in D_2O

a measurable rate and class IV the so-called "hard-to-exchange" hydrogens (cf. Table 2). As shown in Table 1 the relative number of exposed peptide hydrogens is raised in the mercury derivative whereas that of hard-to-exchange hydrogens is decreased. In addition, the value of $t_{1/2}$ in class IV peptide hydrogens has also diminished. Thus the changes in the number and half exchange time of class IV hydrogens run parallel with the changes in thermostability (Table 1) and tryptic digestibility (Szabolcsi, Biszku, 1961) of native and modified aldolase.

Effect of ligands on the conformation and conformational stability of native aldolase and aldolase-$(SHg)_2$

Optical rotatory dispersion was measured in the presence of 50 mM P_i or 70 mM FDP. The Moffitt parameters revealed no significant differences between free enzyme and enzyme-ligand complex, whether native or mercaptidated aldolase was tested (Table 2).

The time course of hydrogen-deuterium exchange carried out in the presence of 50 mM P_i or 70 mM FDP is presented in Fig. 1, curves 3 and 4, and the relative number of peptide hydrogens of different half exchange times is summarized in Table 2. We did not find appreciable difference between the effects of P_i and FDP,

Table 2

Effect of P_i and FDP on the conformation of native and mercaptidated aldolases

Temperature 20°. ORD was measured at pH 7.5, hydrogen-deuterium exchange was performed at pD 7.5. P_i and FDP, if present, were in 50 mM and 70 mM concentration, respectively. The figures represent average values from seven independent experiments

Enzyme	Moffitt parameters		Distribution of peptide hydrogens according to half exchange time							
			class I		class II		class III		class IV	
	$-a_0$	$-b_0$	$t_{1/2}$ min	%*	$t_{1/2}$ min	%	$t_{1/2}$ min	%	$t_{1/2}$ hour	%
Native aldolase	58	160	<1	50	12.5	4	44.7	9	53.5	37
Native aldolase + P_i or FDP	60	157	<1	41	11.6	7	48.1	9	56.4	43
Aldolase-$(SHg)_2$	65	140	<1	56	—	0	29.1	14	41.3	30
Aldolase-$(SHg)_2$ + P_i or FDP	65	140	<1	55	10.7	4.3	38.7	9.3	40.5	31.4

* Zero time value = 0.58 was taken as 100% (cf. Methods).

therefore the plots represent average values. In the aldolase-P_i or aldolase-substrate complex the number of class I peptide hydrogens decreased and that of hard-to-exchange peptide hydrogens increased, which is an indicator of higher conformational stability.

These data are in agreement with our earlier finding according to which saturation of native aldolase with P_i confers great stability to the enzyme at 58° and pH 7.5 (Szajáni et al., 1970).

On the other hand no appreciable differences were found in the rate of hydrogen-deuterium exchange of aldolase-$(SHg)_2$ and its complexes with P_i or FDP, although marked differences were observed in their heat stabilities.

Heat stability was tested by determining the first order rate constant of inactivation at 58° and pH 7.5 in the absence and presence of P_i or FDP. Protection has been defined as $\Delta k = k_E - k_m$, where k_E is the first order rate constant of inactivation of free enzyme and k_m is that measured at a given concentration of ligand.

Both P_i and FDP increased the heat stability of aldolase-$(SHg)_2$. In the case of P_i, the plot $1/\Delta k$ vs. $1/P_i$ gave the downward concave curve shown in Fig. 2, which indicates the existence of two kinds of P_i-binding sites with different disso-

ciation constants.* By assuming that the P_i-binding sites are independent of each other and the stabilizing effects are additive, the phenomenon can be described by equation 1 (Szajáni et al., 1970).

$$\Delta k = (k_E - k_{EP}) \frac{[P_i]}{K_{EP} + [P_i]} + (k_{EP} - k_{PEP}) \frac{[P_i]}{K_{EP} + [P_i]} \times$$
$$\times \frac{[P_i]}{K_{PE} + [P_i]} + (k_E - k_{PE}) \frac{K_{EP}}{K_{EP} + [P_i]} \times \frac{[P_i]}{K_{PE} + [P_i]} \qquad (1)$$

where EP and PE stand for the complex of the enzyme with tightly and loosely bound P_i, respectively, and PEP stands for the enzyme-P_i complex in which both binding sites are occupied. K_{EP} and K_{PE} are apparent dissociation constants.

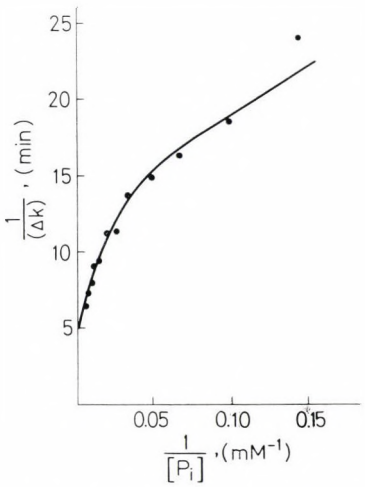

Fig. 2. Effect of P_i on the heat inactivation of aldolase-$(SHg)_2$ at 58° and pH 7.5. Double reciprocal plot of equation (1). $\Delta k = k_E - k_m$, k_E = rate constant of inactivation of the free enzyme; k_m = rate constant of inactivation at P_i concentrations indicated on the abscissa. The solid line is the theoretical curve calculated with the constants listed in Table 3

The best fit for the experimental points of Fig. 2 was obtained with the values listed in Table 3. As shown in our earlier paper (Szajáni et al., 1970) P_i also protected native aldolase and for comparison these constants are included in Table 3. It is clear that binding of P_i stabilizes the conformation of aldolase-$(SHg)_2$ although saturation with the ligand does not confer full stability at 58° and pH 7.5. Since the values of K_{EP} and K_{PE} remained unchanged in aldolase-$(SHg)_2$, the loosening of the structure caused by mercaptidation did not damage the P_i-binding sites.

* Ginsburg and Mehler (1966) have shown by equilibrium dialysis that aldolase contains two kinds of specific P_i-binding sites.

Table 3

Effect of ligands on the heat stability of native and modified aldolases at pH 7.5 and 58°

E denotes free enzyme, *EP* and *PE* stand for the complex of the enzyme with tightly and loosely bound P_i, resp., *PEP* stands for the enzyme-P_i complex in which both binding sites are occupied. FDP stands for equilibrium mixture of substrates (cf. text), K_E was determined in 0.1 M Tris buffer

Enzyme	Rate constants of inactivation, k × 10, (min^{-1})					Apparent dissociation constants (mM)		
	k_E	k_{EP}	k_{PE}	k_{PEP}	k_{FDP}	K_{EP}	K_{PE}	K_{FDP}
Aldolase-$(SHg)_2$	2.75	1.95	0.77	0.55	0.55	7.5	250	0.143
Native aldolase	1.5*	0.3*	0.0*	0.0*	0.0	7.5*	200*	0.143

* Szajáni et al., 1970.

FDP protects both native and mercaptidated aldolases at much lower concentrations than P_i. In both cases the FDP-dependence of protection is a single saturation curve as shown in the double reciprocal plot of Fig. 3. As mentioned in Methods the effect of FDP has been studied in equilibrium mixtures, the values given on the abscissa of Fig. 3 refer to FDP concentrations at zero

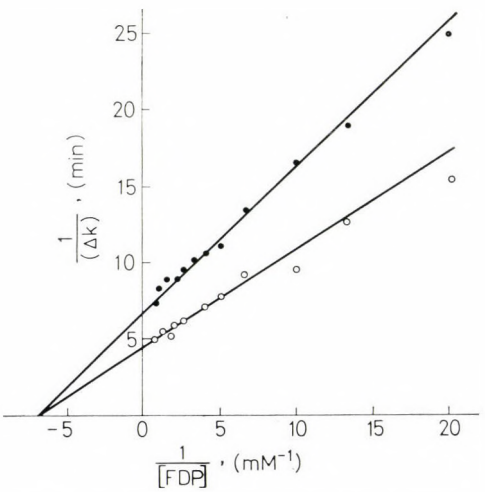

Fig. 3. Effect of FDP on the heat inactivation of native aldolase and aldolase-$(SHg)_2$ at 58° and pH 7.5. Double reciprocal plot of equation (2). Δk = as in Fig. 2, but k_m measured in the presence of FDP. The experiments were carried out in the equilibrium mixtures of substrates, FDP concentrations given on the abscissa are the values at zero time preincubation. The solid lines represent theoretical curves calculated with the constants listed in Table 3, ○, native aldolase, ●, aldolase-$(SHg)_2$

time preincubation. Table 3 summarizes the constants determined graphically from equation (2) (Südi, 1970).

$$\Delta k = (k_E - k_{ES}) \frac{[S]}{K_S + [S]} \tag{2}$$

where k_E and k_{ES} are the first order rate constants of inactivation of free enzyme and enzyme-substrate complex, respectively, and K_S is the apparent dissociation constant, which in the present case is related to the equilibrium mixture of FDP, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate.

The differences observed in the protective effects of FDP and P_i on the heat stability of aldolase-$(SHg)_2$, are probably due to the presence of an equilibrium mixture of substrates, when the probability of both P_i-binding sites being simultaneously occupied is greatly increased.

Our results show that blocking of 2 thiol groups per subunit of aldolase provokes both a marked loosening in the structure and a decrease in the conformational stability of the protein. However, the conformational changes do not damage the specific ligand-binding sites of the enzyme as shown by the unchanged apparent dissociation constants of aldolase-$(SHg)_2$-ligand complexes. This phenomenon might account for the full activity of the mercury derivative.

On the other hand, binding of substrate or P_i to either native or mercaptidated aldolase does not seem to induce changes in the overall conformation of the protein, but it influences the conformational stability of the enzyme. As concluded from studies on the mechanism of hydrogen-deuterium exchange in proteins (Závodszky, 1971), binding of substrate or P_i decreases some of the segmental non-cooperative motions of regions located near the surface of the molecule. This in turn reduces the freedom of movement of internal regions, which manifests itself in an increased conformational stability of the whole protein, without detectable changes in the overall conformation.

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Studies on the Relationship between Quaternary Structure and Enzymatic Activity of Rabbit Muscle Aldolase

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Dedicated to Professor A. E. Braunstein in commemoration of his 70th birthday

1. The specific activity of rabbit muscle aldolase was constant between 0.2 and 70 μg/ml protein concentrations. The first order rate constant of heat inactivation of the enzyme did not change between 0.01 and 1 mg/ml concentrations. These findings suggest that tetrameric aldolase does not dissociate even at low concentrations used in activity assays.

2. Aldolase monomers obtained by direct acylation are markedly unfolded as indicated by gel-filtration studies.

3. A two-step chemical modification of aldolase, successive acylation with 2,3-dimethylmaleic anhydride and maleic anhydride, is described. By this means stable monomers whose active centre is presumably free from blocking agent could be produced, but no enzyme activity was recovered. The results are discussed with regard to the relationship between quaternary structure and enzyme activity.

Introduction

Rabbit muscle aldolase (Fructose 1,6-diphosphate: D-glyceraldehyde-3-phosphate lyase, EC 4.1.2.13) is a tetrameric enzyme of molecular weight 158 000 (Kawahara, Tanford, 1966; Závodszky, Biszku, 1967) and consists of four identical or nearly identical subunits (Chan et al., 1967; Lai, 1968; Sajgó, 1971). Each subunit bears one active centre and the subunits within the tetramer seem to be catalytically independent of one another, as shown by hybridization experiments (Meighen, Schachman, 1970; Penhoet, Rutter, 1971). It has recently been claimed (Masters, Winzor, 1971), in fact it is implied in most relevant studies, that the tetramer is the active species.

Nevertheless, with regard to the relationship between catalytic activity and state of aggregation the examination of the following two interrelated questions

Abbreviations: FDP = D-fructose 1,6-diphosphate; EDTA = ethylenediamine tetraacetate; DMMA = 2,3-dimethylmaleic anhydride; DMM- = 2,3-dimethylmaleyl-; MA = maleic anhydride.

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appears to be warranted: 1) Is indeed the tetramer the only enzymatically active form present under the conditions of activity assay? — and 2) If it is, does quaternary constraint between subunits adjust the proper steric structure of the active site or are subunits self-sufficient catalytic entities clustered together for some other reason?

The first question is related to the possible dissociation of tetrameric aldolase. In general, it may be difficult to establish the aggregational state of the smallest active species of an oligomeric enzyme, because exact molecular weight determinations by physical-chemical methods cannot be readily performed on the microgram per ml scale, i.e. at enzyme concentrations used in activity assays. As intersubunit bonds are usually of non-covalent nature, it follows that dissociation must occur at appropriately low enzyme concentrations. Little is known about aldolase in this respect. Preliminary experiments of Kawahara and Tanford (1966) seemed to indicate the dissociation of aldolase tetramer into dimers below 0.2 mg/ml protein concentration. Bernfeld et al. (1965) reported that aldolase was progressively inactivated with increasing dilution in the 10 to 0.2 μg/ml protein concentration range and explained their results by assuming that aldolase dissociated into inactive monomers.*

As to the second question, we have been reduced to mere speculations for long. Recently Chan (1970) described that aldolase monomers covalently bound to a polysaccharide matrix are capable of displaying enzyme activity. Although this finding suggests that quaternary constraint is not needed for the catalytic function, it carries the uncertainty that the solid matrix may serve as a support for the isolated subunit.

In the present work we made an attempt to tackle both questions outlined above. Our experiments suggest that rabbit muscle aldolase does not undergo spontaneous dissociation around protein concentrations used in the activity measurements. For the problem of quaternary constraint we designed a novel approach: we performed a two-step acylation of the enzyme reversibly protecting the active centre from modification; in this way inactive monomers were produced.

Materials and methods

Rabbit muscle aldolase was prepared and three times recrystallized according to the method of Taylor et al. (1948). The enzyme solutions were freed from ammonium sulfate by gel-filtration on a Sephadex G-50 column equilibrated with the appropriate buffer.

Protein concentration of aldolase solutions was determined spectrophotometrically at 280 nm, by using the extinction coefficient $E_{280}^{mg/ml} = 0.74$ (Biszku et al., 1964).

Aldolase activity was assayed either by the dinitrophenylhydrazine method

* The terms "monomer" and "dimer" will be used to denote the corresponding dissociated forms of aldolase, whereas the term "subunit" will designate protomers within the tetramer or dimer.

of Swenson and Boyer (1957) at 30° or by the hydrazine test of Jagannathan et al. (1956) at 25°, in the following reaction mixtures. *Dinitrophenylhydrazine method:* FDP, 20 mM; hydrazine sulfate, 125 mM; aldolase about 20 μg/ml, in 0.05 M Tris.HCl buffer, pH 7.5. *Hydrazine test:* FDP, 1.7 mM; hydrazine sulfate, 10 mM; aldolase, about 4 μg/ml, in 0.05 M Tris.HCl−0.3 mM EDTA buffer, pH 7.5.

Specific activity was calculated a) in the DNFH method according to Swenson and Boyer (1957) from the relationship: S.A. = $\Delta E_{520}^{10\text{min}}$ divided by the absorbance of the enzyme at 280 nm in the test system; b) in the hydrazine test from ΔE_{240}/min (corrected for the blank reaction without enzyme) divided by aldolase concentration, in mg/ml, in the assay mixture. The specific activity of aldolase preparations varied from 100 to 140 as calculated according to method a), which corresponded to a variation of 32 to 45 in method b).

FDP concentration was determined spectrophotometrically in a coupled system containing excess of aldolase, glyceraldehyde-3-phosphate dehydrogenase, triose phosphate isomerase, NAD and sodium arsenate, by using the extinction coefficient for NADH, $\varepsilon_{340} = 6220$ (Horecker, Kornberg, 1948).

2,3-Dimethylmaleic anhydride was prepared from succinic anhydride and pyruvic acid as described by Harkes (1961). The white crystalline product was re-crystallized from benzene–petroleum ether mixture. Yield (with respect to pyruvic acid): 9.2%. Melting point: 90−92°.

Maleic anhydride, succinic anhydride, γ-aminobutyric acid and fructose-1,6-diphosphate (Ba salt, about 80% pure) were procured from Reanal (Budapest). Acrylamide and bisacrylamide were purchased from Fluka A. G. Sephadex gels were obtained from Pharmacia (Uppsala). All other chemicals were commercial preparations of reagent grade.

Maleylation, dimethylmaleylation and succinylation of aldolase were carried out in a Radiometer pH-stat, starting volume: 10 ml. Succinylation was carried out according to Meighen and Schachman (1970) at 8 mg/ml aldolase concentration in 0.05 M Tris.HCl, pH 8.0, at room temperature. Maleylation and dimethylmaleylation were run at 0°. Maleylation in the one-step acylation procedure was performed at about 3 mg/ml aldolase concentration in 0.02 M Tris.HCl, 1 mM EDTA at pH 8.5. The pH was kept at 8.5 by the addition of 1 N NaOH. MA and DMMA were freshly dissolved in acetone and 0.1−0.2 ml was added to the aldolase solution. Succinic anhydride was added in the solid state. The reaction was completed when alkali consumption ceased. Calculations were based on 25 lysyl residues per subunit.

The number of bound maleyl groups was determined spectrophotometrically by using the extinction coefficients of ε-N-maleyl-lysine, $\varepsilon_{250} = 3360$ and and $\varepsilon_{280} = 308$ (Butler et al., 1969) after gel-filtering the maleylated samples.

The number of bound DMM-groups was also determined spectrophotometrically with the aid of the extinction coefficient for the N-dimethylmaleyl group $\varepsilon_{250} = 2720$. At 280 nm this species has negligible absorption ($\varepsilon < 30$). These values were arrived at by measuring the absorption of N-dimethylmaleyl-γ-aminobutyric acid. This compound can be regarded as an analogue of ε-N-dimethylmaleyl-lysine.

Polyacrylamide gel-electrophoresis was performed according to the method of Hedrick and Smith (1968) at pH 8.5.

Gel-filtration was carried out on a Sephadex G-100 column (0.9 × 130 cm) equilibrated with 0.05 M TrisHCl, 0.4 M NaCl, 1 mM EDTA buffer, pH 7.5, at 5 °. The column was calibrated with blue dextran (mol. wt.: 2 000 000), serum albumin (mol. wt.: 68 000) and pepsin (mol. wt.: 36 000).

Molecular weight was determined in a MOM G-312 ultracentrifuge at 4° in 0.05 M borate buffer, pH 8.0, by the high speed sedimentation equilibrium technique of Yphantis (1964).

Results and discussion

1) The question of spontaneous dissociation of aldolase

The dependence of enzyme activity on protein concentration was examined. As shown in Table 1, the specific activity of the enzyme was practically constant between 70 and 0.2 μg/ml concentrations. Thus, we could not confirm the results of Bernfeld et al. (1965): apparently, under our conditions dissociation into inactive monomers does not occur.

Table 1

Specific activity of aldolase at different enzyme concentrations

Activity was assayed by the DNFH-method at pH 7.5 and 30°.
Reaction time varied between 0.5 and 90 min depending on enzyme concentration

Concentration μg/ml	Specific activity*	Concentration μg/ml	Specific activity*
67.5	128 ± 8	1.7	127 ± 12
33.8	123 ± 8	0.8	114 ± 10
16.8	120 ± 7	0.4	112 ± 11
8.4	134 ± 13	0.2	119 ± 10
3.3	130 ± 10		

* The values are the averages of 4 measurements.

The dependence on protein concentration of the susceptibility to heat denaturation of an oligomeric protein may also provide information about the aggregational state of the oligomer in question. It is reasonable to assume that different aggregational forms are denatured at different rates. Fig. 1 shows the time course of heat inactivation of aldolase at 55°, at 1.0, 0.1 and 0.01 mg/ml enzyme concentrations. Heat inactivation follows first order kinetics down to about 10% residual activity and can be characterized by a single rate constant, $k = 2.2 \pm 0.2$ hour^{-1}, at all the three enzyme concentrations.

The above results seem to suggest that tetrameric aldolase does not undergo dissociation to any appreciable extent in the concentration range examined. Of course, the experiments do not exclude this possibility. However, our data could only be reconciled with such a, rather improbable, dissociating aldolase model in which the enzymatic activities, as well as the heat stabilities, of all existing aggregational forms are the same.

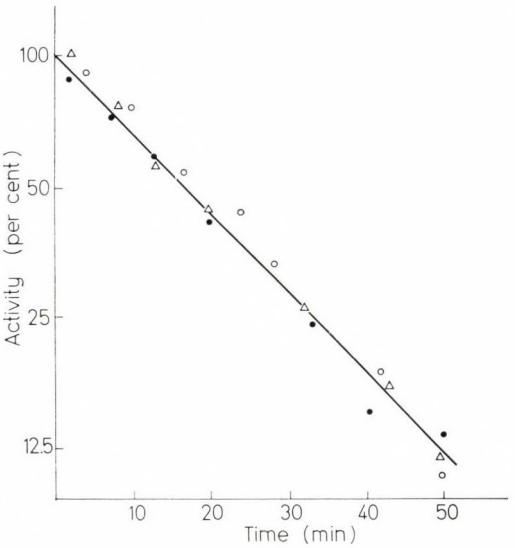

Fig. 1. Heat inactivation of aldolase at different enzyme concentrations. Aldolase solutions in 0.2 M Tris.HCl, 10 mM EDTA buffer, pH 7.5, were incubated at 55 ± 0.1°. Activity was assayed by the dinitrophenylhydrazine test (cf. Methods). ●: 1.0 mg/ml; △: 0.1 mg/ml; ○: 0.01 mg/ml aldolase concentrations

2) Production of aldolase monomers by acylation

(i) Gel-filtration of aldolase monomers produced by one-step acylation. It has been known from the work of Sia and Horecker (1968) and Meighen and Schachman (1970) that aldolase can be dissociated into monomers by extensive maleylation or succinylation of lysyl-ε-NH_2 groups. The subunits obtained by these authors were enzymatically inactive, since most probably the lysyl side chain that forms Schiff-base with the substrate (Grazi et al., 1962) is among the first residues blocked by these reagents. It seemed probable that the tertiary structure of these subunits is also markedly changed, though Sia and Horecker (1968) claimed that the subunits produced by maleylation were still compact globules. We maleylated and succinylated aldolase to various extents as described by the respective authors, and determined the molecular weight both by ultracentrifugation and gel-filtration on a Sephadex G-100 column. If a sufficient amount of anhydride was added (18-S and 40-M) a molecular weight of 37 000 ± 3 000 could be determined in

the ultracentrifuge. However, as shown by the elution profiles in Fig. 2, the presence of monomers is indicated by a shoulder on the tetramer peak and *vice versa*, and monomers are eluted at a position corresponding to a molecular weight higher than that of a hypothetic aldolase dimer. The great apparent molecular weight indicates that these monomers are unfolded to a considerable extent.

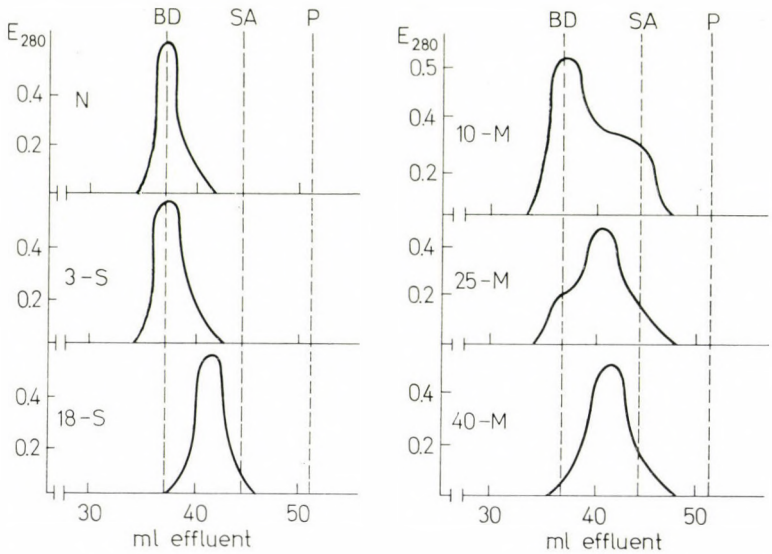

Fig. 2. Gel-filtration elution profiles of native, succinylated and maleylated aldolase samples. Sephadex G-100 column (0.9 × 130 cm) was loaded with 2 ml of solution containing about 4 mg protein. The vertical broken lines indicate the elution volumes of BD = blue dextran, SA = serum albumin and P = pepsin. N = native aldolase; 3-S and 18-S = aldolase samples treated with 3 and 18 moles of succinic anhydride, respectively, per lysyl residue

(ii) Production of aldolase monomers by two-step acylation: successive dimethylmaleylation and maleylation. In light of the foregoing, to produce by acylation monomers whose active centre is free from blocking agent, we applied a two-step modification procedure. 2,3-Dimethylmaleic anhydride and maleic anhydride were chosen as the first and second acylating agent, respectively. The former compound has been reported by Dixon and Perham (1968) to react with NH_2-groups around pH 8.5 and the DMM-group is readily cleaved off in a mild acidic medium, where the N-maleyl bond is not yet affected. The quantities of reagents and the reaction conditions were selected by the aid of an optimalization procedure.

The results of a typical set of experiments are illustrated in Table 2 and Fig. 3. In the first step most of the surface-exposed lysyl-ε-NH_2 groups, among them the Schiff's base-forming lysine, were acylated with DMMA [$(DMM)_9$-aldolase]. [According to Meighen and Schachman (1970) there are 10 lysyl residues per subunit on the surface of aldolase molecule.] This modification did not change

Table 2

Production of aldolase monomers by two-step acylation

Treatment[a]	Activity[b] per cent	K_m of FDP $\times 10^5$, M	Mol. weight[c]	Number of bound[d] groups per subunit	
				DMM	maleyl
1. None	100	3.2	160 000	—	—
2. DMMA	5	—	158 000	8.6	—
3. DMMA + deacylation	60	2.9	156 000	4.8	—
4. DMMA + MA + deacylation	0.1	—	47 000	4.8	5.6

[a] Treatments: DMMA: 20 moles of DMMA per mole lysyl residue were added to a 1 mg/ml aldolase solution in 0.05 M borate buffer, pH 8.5. MA: 40 moles of MA per mole lysyl residue were added immediately after DMMA-treatment. Deacylation consisted of two steps: (i) Acid treatment: β-mercaptoethanol was added (10 mM) and the pH of the solution was adjusted to 4.5 with 0.1 M H_3PO_4 at 0°. The mixture was incubated for 20 min. (ii) Neutralization and hydroxylamine-treatment: The pH was adjusted to 7.5 with 1 M borate, pH 10.5, and the solution was incubated at 22—24° for 2 hours, then hydroxylamine was added (0.8 M, pH 7.5) and the mixture was incubated at room temperature for 15 min followed by incubation at 4° overnight. Hydroxylamine treatment was applied to split possible O-acyl bonds.

[b] Measured by the hydrazine-test.

[c] Determined by high-speed sedimentation equilibrium analysis.

[d] Determined spectrophotometrically by the aid of extinction coefficients given in Methods; aldolase samples subjected to the deacylation procedure without prior acylation served as control. Hydrolyzed (excess) reagent was removed by gel-filtration on a Sephadex G-50 column equilibrated with 0.2 M borate, 1 mM EDTA buffer, pH 8.5. As maleyl and DMM-groups could not be distinguished spectrophotometrically, in line 4 it was assumed that the same number of DMM-groups were bound to the enzyme as in line 3. The latter value was well reproducible (4.8 ± 0.3).

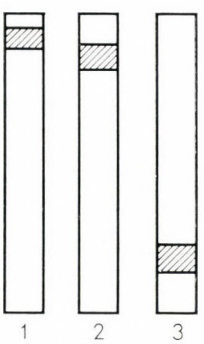

Fig. 3. Polyacrylamide gel-electrophoretic pattern of native and modified aldolase samples. 1: native aldolase; 2: DMMA-treated and reactivated aldolase; 3: aldolase monomers obtained by the two-step acylation procedure. For explanation see the text

the state of aggregation of the enzyme. If $(DMM)_9$-aldolase was subjected to mild acid and hydroxylamine treatment 60% enzyme activity was recovered; the reactivated species was also a tetramer, had the same K_m for FDP as native aldolase, but still contained about 4–5 bound DMM-groups per subunit [$(DMM)_5$-aldolase]. Obviously, in the reactivated species the substrate-binding lysyl residue must be free. If, as the second step, DMMA-treatment was immediately followed by maleylation, against which the substrate-binding lysine was protected by the DMM-group, and then the acid-hydroxylamine treatment was applied, the product was a monomer (mol. wt.: 47 000), which contained in addition to the 4–5 DMM-groups 6 maleyl residues per subunit [$(DMM)_5$-M_6-aldolase]. Dissociation into monomers is also indicated by the fast migration in the gel (Fig. 3).

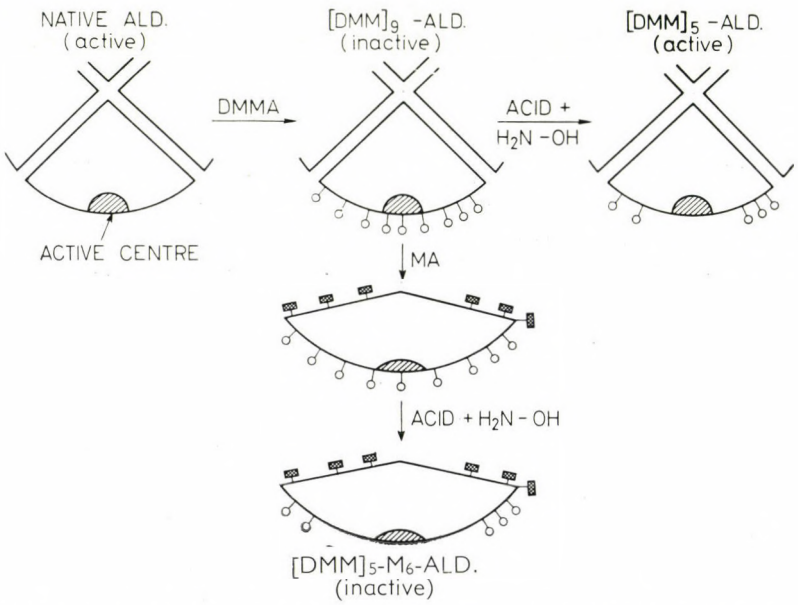

Fig. 4. Proposed scheme of the changes occurring in the two-step acylation procedure. A sector of the circle stands for an aldolase subunit. ⚲ = DMM-group; ▒ = maleyl group. For explanation see the text

The structural changes we assume to occur during this combined modification are schematically depicted in Fig. 4. Dimethylmaleylation does not change the state of aggregation of the enzyme. However, if treatment with DMMA is followed by maleylation amino acid side chains essential for subunit association become blocked. Consequently, dissociation into monomers occurs with the subsequent reaction of further NH_2-groups that became accessible on the contact surfaces thus exposed. By the deacylation procedure the DMM-groups from the active centre are presumably removed. In fact, it is assumed merely by analogy that during deacylation the same DMM-groups are removed before and after maleylation. If this, fairly reasonable, assumption is accepted, then two alternative

explanations can be offered for the complete lack of enzyme activity in the final product, $(DMM)_5$-M_6-aldolase:

a) The insertion of maleyl groups hinders the association of monomers and in the lack of quaternary constraint the proper conformation of the active site, as well as of the whole subunit, cannot be formed.

b) Alternatively, it is not the lack of intersubunit contacts but rather the direct effect of introduced groups that leads to the "collapse" of the subunit with the concomitant deformation of the active centre.

Indeed, we have no basis to reject either interpretation. We only should like to recall that the necessity of quaternary constraint for enzyme activity (explanation *a*) can only be corroborated if a variety of similar approaches lead to the same result.

We wish to point out that the two-step modification technique described in this paper may prove a useful means of studying the role of contact surfaces in oligomeric proteins or even in higher molecular complexes.

The authors wish to express their sincere gratitude to Professor F. B. Straub for his continued interest and to Dr Gertrud Szabolcsi for the helpful discussions. Thanks are due to Dr P. Závodszky for carrying out the sedimentation analyses.

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Studies on the Effect of pH on the Structural and Functional Properties of Pancreatic Amylase

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1. As shown by difference spectrophotometric investigations increasing numbers of tryptophyl and tyrosyl side chains were released from the hydrophobic interior of the amylase (EC. 3.2.1.1) upon lowering the pH. In strongly acidic solutions, at pH 2, all the sixteen tyrosines became accessible to the external solvent medium, whereas a significant number of tryptophans, about seven out of total seventen remained buried.

2. The perturbation difference spectra of the enzyme induced by the binding of maltose appeared to be unchanged over a broad pH range, between pH 4.5 and 8.5, while the hydrolysis of substrate by the enzyme has shown a different pH-dependence.

3. Maltose in 29 mM concentration decreased the rate of acid denaturation of the enzyme, which suggests that a temporary interaction may exist between enzyme and maltose also at acidic pH.

Since the substrates of polysaccharide-hydrolysing enzymes are high molecular weight compounds, it is reasonable to suppose, both on the basis of analogy with lysozyme (Blake et al., 1967) and on the basis of kinetic and binding studies on amylase (Loyter, Schramm, 1966; Robyt, French, 1970), that a relatively large area, i.e. a great number of side chains, is involved in the binding of substrate by the amylase (α-1,4-glucan-4-glucanohydrolase, EC. 3.2.1.1) molecule.

In a previous paper (Elődi, Móra, 1972) we reported that the end-product of α-amylase action, maltose, can be bound to a tryptophyl side chain of the enzyme that is very probably responsible for the binding of the substrate, amylose. We concluded that binding of maltose altered the interaction between the maltose-binding tryptophyl side chain and some charged groups.

In the present work we studied the effect of pH on the structure and stability of amylase and on the binding of maltose by the enzyme in order to reveal some structural features of the enzyme-product (competitive inhibitor) interaction.

Materials

Porcine pancreatic amylase was isolated and twice recrystallized according to Hatfaludi et al. (1966). The crystal suspension was stored in 1 mM calcium chloride at 4 °C and was treated weekly with diisopropyl fluorophosphate to

prevent proteolytic degradation. A stock solution was prepared by dissolving the enzyme in 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM calcium chloride. Protein concentration was determined spectrophotometrically in Tris-HCl buffer, pH 7.4, by using the extinction coefficient $A_{280}^{1\%} = 24$. All calculations were based on a molecular weight of 52 000 (Závodszky, Elődi, 1970; Cozzone et al., 1970). Enzyme activity was assayed at 25 °C by measuring the change in iodine-starch colour according to Smith and Roe (1949). The specific activity of enzyme preparations varied between 4500 to 5500 Smith – Roe units per mg protein as measured at 37 °C.

Soluble starch was purchased from Merck (GFR). Polyethylene glycol (Carbowax 400) was a product of Carlo Erba Co. (Italy). Maltose was obtained from Reanal (Hungary). All other reagents were commercial products of reagent grade.

The pH of amylase samples was adjusted with the following solutions: pH 1.5 to 3.0 : 0.1 M KCl-HCl, pH 3.0 to 6.0 : 0.1 M acetic acid–sodium acetate or 0.1 M formic acid–sodium formate, pH 5.6 – 8.0 : 0.1 M sodium dihydrogen phosphate–sodium hydrogen phosphate, pH 6.0 to 9.0 : 0.1 M Tris-HCl, pH 8.0 to 10.0 : 0.1 M glycine-HCl or 0.1 M sodium borate-HCl. Below pH 9.5 all solutions contained 1 mM calcium chloride.

Methods

Difference spectra were recorded between 260 and 330 nm in an Opton DMR 21 (Oberkochen, GFR) recording spectrophotometer in 1 cm silica cells thermostated at 25 ± 0.1 °C. Protein concentration was about 7.5 to 10 μM (400 to 500 μg per ml). The change in the exposure of chromophores due to the unfolding of polypeptide chain at acidic pH was calculated by using the absorption differences found earlier with amylase (Elődi, Krysteva, 1970). These values are for a tryptophyl side chain $\Delta\varepsilon_{293} = 2650$, and $\Delta\varepsilon_{287} = 1540$, for a tyrosyl side chain $\Delta\varepsilon_{287} = 650$. The absorption difference for tyrosyl side chain at 279 nm was derived from solvent perturbation and denaturation difference measurements and it was $\Delta\varepsilon_{279} = 485$.

The number of chromophores accessible to the solvent was determined in tandem cells by the solvent perturbation method: difference spectra were recorded in 20% polyethylene glycol against polyethylene glycol-free solutions. For the calculations the molar absorption differences given by Herskovits and Sorensen (1968) were used. The protein concentration in the solvent perturbation experiments was about 15 μM which gave an absorbancy of about 1.9 at 280 nm, so that slit width never exceeded 0.2 mm due to the properly selected photomultiplier gain adjustment.

The pH dependence of maltose-induced perturbation difference spectra of amylase was studied in the following way. To a stock solution of amylase dissolved in 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM calcium chloride maltose was added and the samples were diluted tenfold with different buffers between

pH 2.5 and 6.0 to a 15 μM protein concentration and 29 mM maltose concentration. The difference spectra of amylase solutions containing maltose were recorded against amylase references of the same pH without maltose.

The pH of the solutions was checked by a Radelkisz OP 205 type precision pH meter with an accuracy of ± 0.02 pH units at 25 °C. Molecular weight determinations were carried out in a MOM G-3131 type ultracentrifuge equipped with Rayleigh interferometer optics. Light scattering measurements were performed with a Brice-Phoenix S-2000 type photometer.

Results and discussion

Effect of pH on the ultraviolet absorption of amylase

Porcine pancreatic amylase contains 17 tryptophyl (Előd i, Krysteva, 1970), and 16 tyrosyl (Straub et al., 1970) residues, which renders the enzyme suitable for spectrophotometric investigations. It was of interest to know the behaviour of the enzyme along the pH scale, particularly the alterations of chromophoric groups at different pH-s. Therefore, we studied the changes in the absorption of amylase as a function of pH by difference spectrophotometry.

The difference spectra of 7.5 to 10 μM amylase solutions of pH 1.6 to 5.0 were recorded against a neutral solution at 25 °C and the spectral changes were followed in time. In 0.1 M acetate buffer (pH 4.5 to 3.8) a difference spectrum was observed instantaneously after adding the acidic buffer to the protein. The maxima of this difference spectrum were found at 293 and 287 nm (Fig. 1, curve *a*), which suggests that this optical change is due to the release of tryptophan from the interior of the protein.

Upon further decrease of pH, between pH 3.8 and 2.8, beside the difference spectrum instantaneously formed, additional changes occurred in the spectra recorded at different times (Fig. 1, curves *b* and *c*). The absorption differences at the maxima characteristic of tryptophan (293 and 287 nm) and of tyrosine (287 and 279) were increased during incubation. In addition, a shoulder appeared around 305 nm which is due to the abolishing of interaction between tryptophyl side chain(s) and negative electric charges, which prevailed in the native protein (Ananthanarayanan, Bigelow, 1969). In strongly acidic solutions, below pH 2.8, instantaneous spectral changes were again detected. The shape of the difference spectra, the positions and intensities of the maxima, however, coincided with those observed at higher pH-s after longer incubation.

It is to be mentioned that in acidic solution amylase shows a tendency to aggregate. This is especially pronounced under conditions where the absorption difference appears instantaneously and this may slightly reduce the actual absorption differences. The aggregation of amylase in acidic medium was also confirmed by following the increase in turbidity of the solution by light scattering and by determining the increased molecular weight in the ultracentrifuge. The association of the protein at acidic pH seemed to be a limited process, since under our experimental corditions there was neither opalescence nor precipitate in the solutions.

The number of exposed chromophoric side chains at acidic pH was estimated from the absorption differences at the characteristic wavelengths, i.e. at 293 and 287 nm for tryptophan and 287 and 279 for tyrosine. Fig. 2 shows the pH-dependence of the molar absorption differences at 293 and 279 nm and at the 305 nm shoulder. It can be seen in Fig. 2 that the structure of the protein changes below pH 5. The pH dependence of both $\Delta\varepsilon_{305}$ and $\Delta\varepsilon_{279}$ can be characterized by a theoretical dissociation curve of an apparent pK = 3.4, as calculated from the equation $pH = pK + \log \alpha/1-\alpha$. The pH dependence of absorption changes at 293 and that at 287 nm (not shown in Fig. 2) cannot be described by a single theoretical dissociation curve.

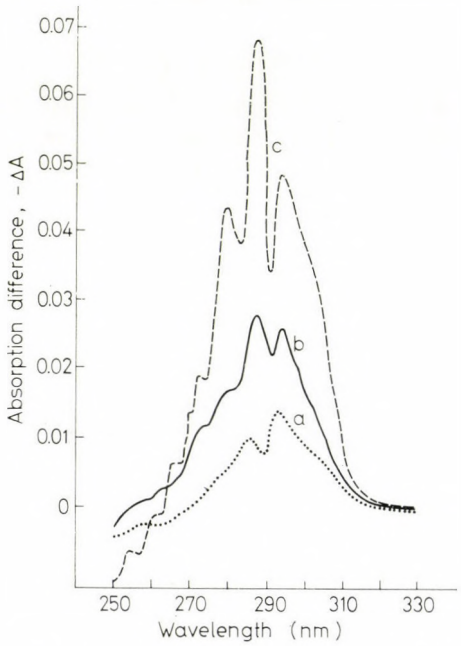

Fig. 1. Denaturation difference spectra of amylase at acidic pH. The spectra of amylase solutions of different pH's were recorded against neutral references in 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM calcium chloride at 25 °C. Curve a — 7.5 μM protein in 0.1 M acetate buffer, pH 3.8 recorded 2 min after the addition of the acidic buffer. Curves b and c — 10 μM protein in 0.1 M acetate, pH 3.0, 15 and 240 min after the addition of acid, respectively

The insert in Fig. 2 shows the number of chromophores released as a function of pH. By decreasing the pH from neutral to pH 4 about 0.7 tryptophyl residues were released with an apparent pK of 4.4. This side chain was probably only partially buried in the native protein at neutral pH. The difference spectrum recorded under these conditions corresponded to curve *a* in Fig. 1. Upon further decrease of pH from 4 to 2 the environment of about two additional tryptophyl and about ten tyrosyl side chains was changed with the concomitant unfolding of the polypeptide chain. The apparent pK of the unfolding was about 3.4.

Table 1

Number of exposed chromophoric side chains in amylase at different pH-s

Calculated from the solvent perturbation difference spectra recorded in the presence of 20% polyethylene glycol against references without polyethylene glycol. (Accuracy of determinations ±5%.)

pH	Number of accessible side chains per mole amylase			
	Tryptophan		Tyrosine	
	total	change	total	change
7.4	6.8	0	7.2	0
4.2	7.5	0.7	6.9	−0.3
4.0	7.9	1.1	8.0	0.8
3.5	8.8	2.0	13.5	6.3
2.3	9.6	2.8	16.7	9.5

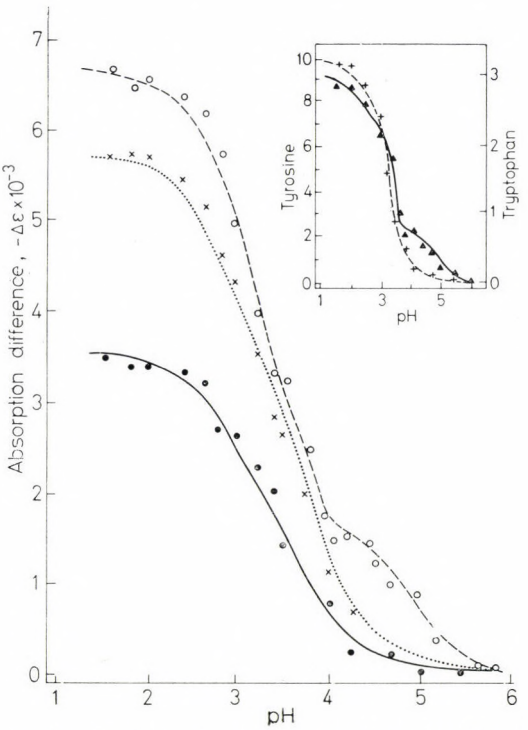

Fig 2. pH-dependence of molar absorption differences of amylase. The absorption differences were measured at 279 (×), 293 (○) and 305 (●) nm with 7.5 μM protein samples between pH 1.6 and 6.0 (see Methods) against a neutral reference solution and the molar absorption differences were plotted against pH. The insert shows the number of tryptophyl (▲) and tyrosyl (+) side chains released from the interior of the protein at different pH-s, calculated from the molar absorption differences of Fig. 2

The number of accessible chromophoric side chains was also determined by solvent perturbation measurements in 20% polyethylene glycol at acidic pH (Table 1). The data presented in Fig. 2 and in Table 1 satisfactorily agree. We can conclude from Table 1 that at pH 2.3 practically all the sixteen tyrosines of the protein are in an accessible position to the solvent medium, whereas somewhat more than half of the tryptophans, only about ten out of seventeen, appeared to be in contact with the external solvent medium. These data suggest that the amylase molecule contains a hydrophobic nucleus and about seven tryptophyl side chains are located in this nucleus, which is stable even at extremely acidic pH.

Effect of pH on the amylase–maltose complex

As we described in an earlier paper (Elődi, Móra, 1972), the difference spectrum of amylase–maltose complex exhibited a sharp maximum at 290 nm. To characterize the pH-stability of enzyme–maltose complex we studied the pH-dependence of the maltose-induced spectral change in the following way. The spectra of amylase solutions containing maltose were recorded against amylase references of the same pH in the absence of maltose, and the molar absorption differences found at 290 nm were plotted against pH. As seen in Fig. 3, the $\Delta\varepsilon_{290}$ values, i.e. the binding of maltose to the enzyme, exhibit a plateau between pH 4.5 and 8.5 and decrease sharply below and above these pH-s.

The pH-dependences of $\Delta\varepsilon_{290}$ and of enzyme activity (Fig. 3) differ markedly. Thus e.g. at pH 5.0 where enzyme activity is only about 10% of the maximum, maltose binding appears to be still complete.

Below pH 4.0 the binding of maltose to amylase could not be followed by measuring the maltose-induced spectral alterations because gross conformational

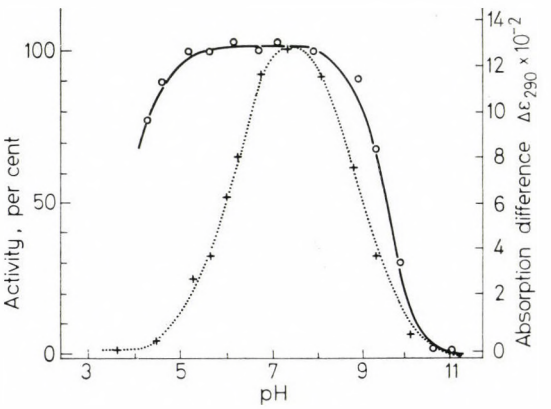

Fig. 3. pH dependence of maltose binding and enzyme activity. The absorption differences at the maximum of maltose-induced difference spectra ($\Delta\varepsilon_{290}$) were recorded at different pH-s with solutions containing 15 μM protein and 29 mM maltose against an enzyme solution of the same pH without maltose (open circles). Amylase activity (+) was calculated from a 10% decrease of the iodine-amylase colour at 25 °C, in the presence of 1 nM enzyme

changes also occurred under these conditions. To decide whether maltose is still bound to the enzyme at acidic pH we studied the effect of 29 mM maltose on the rate of denaturation of amylase. The difference spectra of 7.5 μM amylase solutions between pH 2.5 and 3.5 in the presence and absence of 29 mM maltose were recorded at 25 °C at different times against a neutral reference. The rate of unfolding was calculated from the absorption differences at 279, 287, 293 and 305 nm.

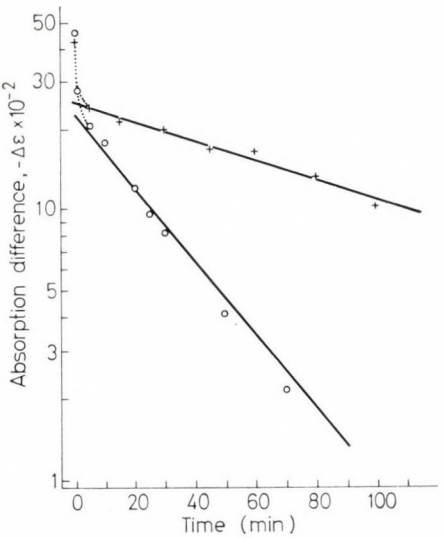

Fig. 4. The rate of denaturation of amylase at pH 3.0. The changes in the absorption of 7.5 μM amylase solution at 287 nm were followed in the absence (○) and presence (+) of 29 mM maltose in 0.1 M acetate buffer, pH 3.0. Temperature 25 °C

Fig. 4 shows the development of absorption differences at 287 nm, which indicates the contribution of both tryptophan and tyrosine to the absorption differences.

It should be noted that the absorption changes of amylase in this pH range take place in two steps. The first one was too fast to be followed in time under our experimental conditions. This step corresponds to the release of practically one tryptophyl side chain (see also Fig. 1, curve *a*).

As seen in Fig. 4, the slow step of unfolding of the enzyme follows first order kinetics. The rate constants calculated are 0.031 and 0.008 $\times$ min^{-1} in the absence and presence of maltose, respectively. It should be pointed out that the rate constants calculated from the absorption changes at the other three wavelengths, i.e. 279, 293 and 305 nm, agree with those found at 287 nm within the limits of experimental error ($\pm 15\%$).

As seen in Fig. 4 the rate of denaturation at pH 3 measured in the presence of maltose is significantly smaller than that in the absence of maltose. Sucrose in 29 mM concentration had no appreciable effect on the rate of denaturation. That maltose influences the rate of denaturation may indicate that an interaction

between maltose and amylase exists even at a relatively low pH. Maltose exerted no detectable effect on the acid denaturation of the enzyme below pH 2.6.

We can conclude that the maltose-tryptophyl side chain interaction in amylase also exists at relatively acidic pH where only a few per cent of the original enzyme activity can be detected. At slightly acidic pH about one tryptophyl side chain is removed from the internal part of the protein ($pK_{app} = 4.4$), which is probably not directly involved in the binding of maltose. Below pH 3.8 amylase is gradually unfolded and the difference spectrum characteristic of maltose binding is also abolished. The interaction between enzyme and maltose, however, seems to exist even at pH 3.0, as indicated by the reduced rate of denaturation by acid in the presence of maltose (Fig. 4). The data suggest (Table 1, Fig. 2) that amylase contains a relatively strong hydrophobic core which is stable even at extremely acidic pH. About 6 to 7 out of the total 17 tryptophyl residues of the enzyme appear to be located in this acid-resistant part of the protein inaccessible to the external solvent medium.

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On the Role of Hydrogen-Bonding System in the Catalysis by Serine Proteases

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Dedicated to Professor A. E. Braunstein in commemoration of his 70th birthday

The probable function of an extended hydrogen-bonding system is discussed. This stabilizes the tetrahedral intermediate and the imidazolium ion formed during the catalysis by serine proteases. In addition to the amino acid residues (serine, histidine and aspartic acid) of the charge relay system proposed previously by Blow, Birktoft and Hartley (1969), the system involves the substrate and other amino acid side and main chains, too. The extended hydrogen-bonding system is a symmetric network capable of acting reversibly, which is a requirement for the proton transfer from the nucleophile to the leaving group, the most important feature of the catalytic mechanism. The proposed functioning of the hydrogen-bonding system is supported by present day evidences based on X-ray diffraction and kinetic measurements.

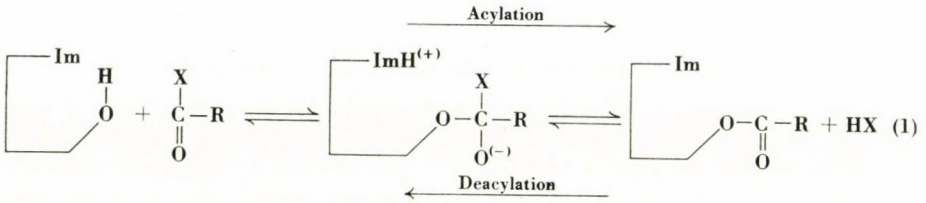
Introduction

One of the most important recent contributions to our knowledge about the mechanism of action of serine proteases comes from X-ray diffraction studies on chymotrypsin (Blow et al., 1969), subtilisin (Alden et al., 1970) and elastase (Shotton, Watson, 1970), which revealed a common hydrogen bond network in these enzymes. It was suggested (Blow et al., 1969) that the hydrogen-bonding system operates as a charge relay system involving the "reactive" Ser-195, His-57 and Asp-102 in the case of chymotrypsin. The corresponding amino acid residues of subtilisin BPN' are Ser-221, His-64 and Asp-32 (Alden et al., 1970). However, the mode of functioning of the charge relay system remained questionable (Polgár, Bender, 1969; Rajender et al., 1971). In this paper, a mechanism of action for the hydrogen-bonding system will be proposed.

The current idea about the catalytic mechanism

The mechanism of action of serine proteases is fairly well understood (Bender, Kézdy, 1965; Blow, Steitz, 1970) and its main features are described by scheme (1).

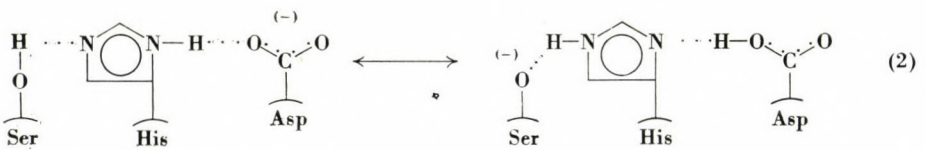
It is seen in scheme (1) that a nucleophilic attack by the hydroxyl group of a serine residue on the carbonyl carbon atom of an ester or amide substrate is catalyzed by a histidine residue as a general base. This leads to the formation



(1)

of a tetrahedral intermediate and imidazolium ion. The intermediate breaks down by general acid catalysis to an acyl-enzyme, an imidazole base and alcohol or amine. The acyl-enzyme is hydrolyzed via the reverse mechanism of acylation. In hydrolysis, the hydroxyl group of a water molecule corresponds to the hydroxyl group of the serine residue.

Scheme (1) does not imply any hydrogen-bonding system. It only indicates that the imidazole group operates as a simple general base and general acid catalyst, although it is known from the aforementioned X-ray diffraction studies that the histidine residue is hydrogen-bonded to the carboxyl group of a particular aspartate residue as seen in scheme (2).



(2)

Discussing the function of the hydrogen bond network, Blow et al. (1969) described it as a charge relay system conducting electrons from the carboxyl group through the imidazole ring to the oxygen atom of the serine residue [scheme (2)]. This implies that the proton of the hydroxyl group of the serine residue is transferred to the $N^{\varepsilon 2}$ atom of the histidine side chain and the proton bound to the $N^{\delta 1}$ atom is transferred to the carboxyl group. In this way the oxygen atom of the serine residue becomes a powerful nucleophile, capable of attacking the carbonyl carbon atom of the substrate. To this passive charge relay system, Rajender et al. (1971) have recently opposed a dynamic mechanism in which conformational changes, triggered by the binding of substrate, alter the basicity of the catalytic groups.

The function of the charge relay system

The idea of the charge relay system is to explain the high nucleophilic reactivity of the serine residue, which is supposed to be due to hydrogen bond formation between the serine and histidine residues. However, it was recently pointed out (Polgár, Bender, 1969) that the high nucleophilicity of the serine residue may not be due to the increase of negative charge on the oxygen atom

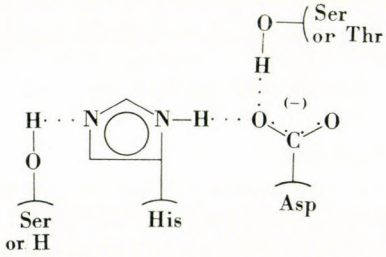
owing to the formation of a hydrogen bond. The formation of a strong hydrogen bond and especially the transfer of the proton from the serine to the histidine side chain would significantly modify the apparent pK of the imidazole ring, in contrast to experimental findings. In fact, in the case of thiol-subtilisin, where the oxygen atom of the serine residue is replaced by a sulfur atom, the proton transfer from the thiol group to the imidazole ring is possible. In this case a hydrogen-bonded ion pair is formed between the mercaptide and imidazolium ions which does not show the pK of the imidazole group, in contrast to the original serine protease (Polgár, Halász, 1971). Another evidence against the importance of the high negative charge on the serine residue in serine proteases is the reaction of the acyl-enzyme with ionizable nucleophiles (Wedler et al., 1970). These experiments show that the ionization of the nucleophile (the serine residue or water) inhibits rather than promotes catalysis. Accordingly, it appears to us that the extremely high nucleophilicity of the serine residue is not at all the consequence of increased negative charge on the oxygen atom, although this notion is widely accepted in the literature. It is the high reactivity only that can be observed and this reactivity may be due to the assistance of the reaction on the serine residue by a unique general base–general acid catalysis to be discussed below. This view is supported by the fact that in some reactions of serine proteases, instead of the serine, the histidine residue is the nucleophile (Ong et al., 1964; Shaw et al., 1965), which would not be possible if the proton of the hydroxyl group were covalently bound to the histidine side chain.

It follows from the foregoing that the role of the hydrogen-bonding system in the catalysis should be more complex than suggested so far. In fact, the imidazole group which is hydrogen-bonded to the aspartate residue catalyzes two successive proton transfers as it is shown in scheme (1). On the basis of the charge relay system, the promotion of the first proton transfer, the general base catalysis could only be explained; the second proton transfer, the general acid catalysis would be inhibited due to the interaction between the imidazolium and the carboxyl groups. The above problem is encountered in the dynamic model of Rajender et al. (1971). Namely, the binding of substrate, which takes place in a previous step, i.e. in the formation of the enzyme substrate complex, is not expected to induce alternate conformational changes for promoting the subsequent general base and general acid catalytic processes during the formation of the acyl-enzyme. This would be even more unlikely in deacylation.

Hydrogen-bonding system in the ground state of the reaction

We have previously suggested (Polgár, Bender, 1969) that the hydrogen-bonding system may play an important role in maintaining the proper steric structure of the active site. At the same time, the hydrogen-bonding system may operate as a reversible system in which proton transfers to and from the $N^{\varepsilon 2}$ atom of the histidine residue can readily occur, if the following two criteria, different from those for the charge relay system, are met.

(1) In addition to the serine, histidine and aspartate residues forming the charge relay system, another serine residue (Ser-214 in chymotrypsin and Ser-33 in subtilisin BPN') hydrogen-bonded to the aspartate residue plays a catalytically important role in the hydrogen-bonding system (scheme 3). This assumption has been made by Freer et al. (1970) on the basis of X-ray diffraction studies. It may



(3)

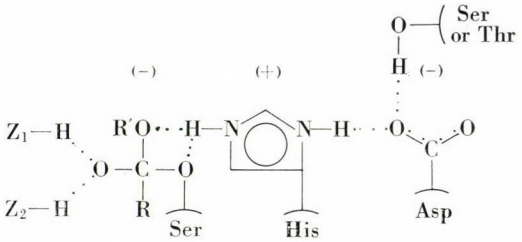
be noted that in the case of Carlsberg type subtilisin this second serine residue is replaced by a threonine residue. Of course, the threonine residue is capable of acting similarly to the serine residue.

(2) Proton transfer between the imidazole and the carboxyl-groups, that is, breaking of the N−H bond and new covalent H−O bond formation are not possible. Bond breaking and bond making would enhance the energy of activation, and since in this case the imidazole, instead of the imidazolium, group would have to donate the proton to the leaving group, the driving force for general acid catalysis would significantly decrease. This implies that only partial electron transfer may take place between the carboxyl and the imidazole groups, as well as between the aspartate and the serine or threonine residues.

Symmetry is an important feature of scheme (3) inasmuch as the hydrogen-bonded pair of the imidazole and the carboxyl groups operates between two hydroxyl groups. In the free enzyme these two hydroxyl groups belong to two different serine residues (the one hydrogen-bonded to the carboxyl group may belong to a threonine side chain); in the acyl-enzyme a water molecule substitutes for the serine residue which takes part in acyl-enzyme formation.

Extended hydrogen-bonding system during catalysis

As the acylation or deacylation reaction proceeds, the imidazole group accepts the proton from the hydroxyl group, and a tetrahedral intermediate with the substrate is formed at the same time [scheme (1)]. In this state of the reaction, the hydrogen-bonding system changes to the form depicted in scheme (4), which



(4)

shows that the hydrogen-bonding system becomes significantly extended during catalysis. The substrate and some amino acid residues are added to the system of scheme (3). The possibility of formation of a bifurcated hydrogen bond between the tetrahedral intermediate and the imidazolium ion was recently put forward (Polgár, 1971). The formation of hydrogen bonds between the carbonyl oxygen atom of the substrate and residues Z_1, Z_2 is supported by X-ray diffraction studies (Henderson, 1970), and kinetic evidence (Williams, 1970). In the case of chymotrypsin the backbone $-NH-$ groups of Ser-195 and Gly-193 provide the hydrogen atoms for the hydrogen bonds (Henderson, 1970).

The mechanism of action of the hydrogen-bonding system can be visualized as follows. When the imidazole group accepts the proton, the strength of the hydrogen bond between the aspartate and the serine residues decreases. At the same time, the hydrogen bond between residues Z_1, Z_2 and the tetrahedral intermediate are gaining strength. When the imidazolium ion releases the proton, the opposite should occur. Such a functioning appears to be akin to that of coenzymes. For example, in group transfer reactions the coenzyme, which contains a conjugated system, is often transformed into an intermediate product having pronounced chemical reactivity due to the existence of high local density of net-electron charges, and great energetical stability due to high resonance energy (Perault et al., 1960). In serine proteases, the intermediate complex of the tetrahedral intermediate and the imidazolium ion should have a pronounced chemical reactivity due to the separation of charges. (It may be noted that in the charge relay system the positive charge of the imidazolium ion is cancelled by the proton transfer to the carboxyl group.) Furthermore, the intermediate complex is stabilized by hydrogen bonds, the substrate becomes a part of a hydrogen bond network rather than that of a conjugated system. The symmetric charge distribution around the positive imidazolium ion also contributes to the stability of the system. This assures an energy barrier for the enzymatic reaction much lower than that of a simple imidazole-catalyzed reaction. Thus the hydrogen-bonded system can be regarded as a "built in" coenzyme. It is possible that some other enzymes also work with "built in" coenzymes, which might have been of significance in evolution.

The reasoning in the previous paragraph implies that the energy level of the extended hydrogen-bonding system should be close to those of the transition states of bond making and bond breaking processes, that is, in the energy profile of the reaction a shallow dip corresponds to the formation of the intermediate system of scheme (4). In this case the intermediate system would have transition state-like properties because its conversion to the transition states involves only a small molecular rearrangement.

The reaction proceeding through the intermediate complex represented by scheme (4) follows a one-encounter type mechanism, which means that two proton transfers occur at one encounter of the reacting groups. This possibility has been raised to explain non-enzymatic organic reactions (Eigen, 1967) as well as the catalysis by chymotrypsin (Bender, Kézdy, 1965). However, in these cases the transfer of *two different* protons takes place in a concerted reaction by the parti-

cipation of one or more water molecules. In the case of serine proteases operating with the hydrogen-bonding system of scheme (4), the *same* proton is transferred through two successive steps. Only this reaction can be regarded as a real one-encounter type (Jencks, 1969). It seems probable that this type of reaction is not unique in enzymology. The proteins are designed to hold together the reacting groups during two reaction steps and this facilitates the one-encounter mechanism.

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The Allosteric Properties of the Isoenzymes of Pig Heart Phosphorylase

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Phosphorylase *b* was prepared from pig heart and after purification it was separated into isoenzymes on a DEAE-cellulose column. By elution with increasing concentrations of glycerophosphate buffer three phosphorylase fractions were separated (in the order of elution: fractions P_1, P_2 and P_3). The allosteric properties of fractions P_1 and P_2 were thoroughly studied by means of kinetic analysis.

G-6-P, AMP and ATP influence differently the isophosphorylases of the heart. Fraction P_3 can be inhibited more by G-6-P and less by ATP than fraction P_1. Fraction P_1, similarly to pig skeletal muscle phosphorylase, cannot be inhibited by G-6-P.

On the basis of its inhibition by high concentrations of G-6-P about 60% of the total phosphorylase in pig heart extracts was identified as fraction P_3.

The allosteric susceptibility of the heart phosphorylase fraction P_3 suggests that under anoxic conditions in vivo this isophosphorylase is capable of catalyzing and controlling glycogenolysis in form *b* as well.

Introduction

Phosphorylase (α-1,4-glucane : orthophosphate glucosyl transferase; EC 2.4.1.1), the limiting enzyme in glycogenolysis, occurs in forms *a* and *b* in the muscle. The conversion of the two forms into each other plays an important role in the regulation of glycogenolysis. Form *b* possesses an enzymatic activity only in the presence of AMP, while form *a* is active also in the absence of AMP. The regulation due to the allosteric activation of phosphorylase *b* by AMP and its inhibition by ATP and G-6-P have an important physiological role, since the actual activity of phosphorylase *b* is determined by the concentration and proportion of these effectors. Morgan and Parmeggiani (1964a, 1964b) have studied the allosteric regulation of phosphorylase *b* under anoxic conditions and assumed that phosphorylase *b* might also play an important role in the mobilization of glycogen when the heart is in an anoxic condition (Morgan, Parmeggiani, 1964a; Oye, 1967). Their experiments were carried out on perfused rat heart and their conclusions were based on the allosteric properties of the phosphorylase *b* of rabbit skeletal muscle. In a number of animal species, however, the myocardium

Abbreviations: G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; Pi, inorganic orthophosphate; AMP, adenosine 5'-monophosphate; ATP, adenosine 5'-triphosphate; EDTA, ethylenediamine tetra-acetate.

may contain not a single phosphorylase, but, in contrast to the skeletal muscle, several phosphorylases.

Yunis et al. (1962) and later Davis et al. (1967) reported in their study on the chromatographic and electrophoretic properties of heart phosphorylases on the properties of one heart isophosphorylase which differed from those of the skeletal muscle. Nevertheless, our knowledge of the allosteric properties of isophosphorylases of the heart and of the differences between these properties is rather scarce and the limited knowledge we have is confined to a single species, the rabbit (Davis et al., 1966).

It may be assumed that the presence of isophosphorylases in the heart has a certain functional significance. It seemed therefore interesting to study the allosteric properties and physiological role of heart isophosphorylases. In earlier experiments we have found that the isophosphorylases prepared from the hearts of different species possess more or less different allosteric properties. The greatest differences were found among the pig heart isophosphorylases. Therefore a detailed kinetic analysis of the biochemical and allosteric properties of these particular isoenzymes seemed to be especially promising. Some of the results have already been reported (Vereb, Csornai, 1970).

Methods

Preparation of phosphorylase b from pig heart

Phosphorylase *b* was purified from pig heart by a modified version of the method of Yunis (Yunis et al., 1962) as follows:

1) Preparation of the extract. – Fresh pig heart was washed with ice-cold water, minced in a meat grinder and homogenized in a 2.5-fold volume of 1 mM EDTA, pH 7.0, in a blade homogenizer for 2 minutes. The homogenate was centrifuged at $1500 \times g$ for 30 minutes in the cold and the supernatant processed.

2) Separation of the pH 5.4 isoelectric precipitate. – The supernatant was adjusted to pH 5.4 with 1 M acetic acid, allowed to stand for 10–30 minutes and then centrifuged at $1500 \times g$ for 30 minutes. The supernatant containing the bulk of the phosphorylase was filtered through folded filter paper and the pH of the filtrate adjusted to 6.8 with solid $KHCO_3$.

3) Precipitation with ammonium sulfate. – 0.8 parts by volume of an ammonium sulfate solution saturated at 20° (pH 7.0) was added to the filtrate. The precipitate formed was allowed to settle for 15 to 20 hours and the supernatant carefully decanted. The precipitate was centrifuged, followed by suspension in an equal volume of 1 mM EDTA – 1 mM Tris-HCl buffer, pH 6.8, and dialysis with repeated changes against the same buffer for about 20 hours.

4) "Heat treatment." – The protein impurities which separated during dialysis were removed by centrifugation and the supernatant containing the phosphorylase was mixed with mercaptoethanol to a final concentration of 0.03 M. The pH was adjusted with 2 M Tris to 8.6. This solution was then incubated at 37° for 60 minutes ("heat treatment"). After cooling the pH of the solution

was adjusted to 6.8 with 1 M acetic acid and the small amount of precipitate formed during heat treatment was removed by centrifugation.

5) Fractionation with ammonium sulfate. – To the clear supernatant a saturated ammonium sulfate solution, pH 6.8, was added until a saturation level of 0.22 was reached. The mixture was allowed to stand for 24 hours at 0°. The precipitate was removed by centrifugation. The ammonium sulfate concentration in the supernatant was raised to a saturation level of 0.34. The precipitate formed upon standing was then collected by centrifugation, dissolved in a minimum volume of 3 mM glycerophosphate – 1 mM EDTA – 3 mM mercaptoethanol buffer, pH 6.8, and dialyzed against the same buffer.

6) DEAE-cellulose chromatography. – The dialyzed phosphorylase solution was chromatographed on a DEAE-cellulose column. The enzyme solution containing about 1 g protein was applied to a 1.8×50 cm DEAE-cellulose (Schuchardt) column equilibrated with a 3 mM glycerophosphate – 1 mM EDTA – 3 mM mercaptoethanol buffer. After washing with 200 ml of the initial buffer on two-step linear gradient elution with glycerophosphate was performed. In the first step 1 liter glycerophosphate with a linearly increasing concentration from 3 mM to 15 mM containing 1 mM EDTA and 3 mM mercaptoethanol was used for elution. In the second step elution was performed with 1 liter of glycerophosphate with a linearly rising concentration from 15 mM to 100 mM and containing 1 mM EDTA and 3 mM mercaptoethanol. The flow rate was 30 to 40 ml per hour. The eluate was collected in 15 ml fractions and in the fractions phosphorylase activity was determined.

7) Concentration of the eluate. – The fractions containing the various isophosphorylases were collected and dialyzed against saturated ammonium sulfate solution, pH 6.8, at $+5°$ until the precipitate settled (24 to 48 hours). The precipitate was concentrated by centrifugation dissolved in a minimum volume of 30 mM glycerophosphate – 1 mM EDTA – 15 mM mercaptoethanol buffer, pH 6.8, and the solution dialyzed against the same buffer until all ammonium sulfate was removed. The isoenzyme preparations thus obtained were treated before use with Norite to remove nucleotides. The phosphorylase obtained by preparing fraction P_1 had a specific activity of 8 units per mg protein, the specific activity of fraction P_3 was 11 units per mg protein.

Preparation of phosphorylase b from pig skeletal muscle

Phosphorylase *b* was prepared from pig skeletal muscle by the method of Fischer and Krebs (Fischer, Krebs, 1958). The preparation (specific activity, 25 units per mg protein) was used without crystallization, since pig phosphorylase *b* does not crystallize in the presence of AMP and Mg.

Measurement of phosphorylase activity

The activities of the phosphorylases were measured by Cori's method (Cori, Illingworth, 1956). The composition of the incubation medium was: 16 mM

G-1-P, 1% glycogen, 10 mM Na-β-glycerophosphate, 0.5 mM EDTA, 7.5 mM mercaptoethanol, pH 6.8, and 1 mM AMP (if not otherwise stated). The quantity ofphospho rylase was chosen in such a way that in the period (usually 10 minutes) of incubation (or of activity measurement) maximum 10 to 15% of the added G-1-P should be converted, that is the reaction should remain linear. Phosphorylase activity was expressed in units, one unit of enzyme is defined as the amount of phosphorylase which liberates 1 μmole inorganic P from G-1-P in 1 minute.

Glycogen was prepared from rabbit liver and the product treated with Norite to remove nucleotides (Helmreich, Cori, 1964). Protein was determined by the method of Gornall et al. (1949).

Results

Chromatographic separation of pig heart isophosphorylases. — Inhibition of enzyme activity by G-6-P and ATP

Chromatography of the purified heart phosphoryiase preparation on DEAE-cellulose column gave the chromatographic pattern shown in Fig. 1.

Fig. 1 shows that pig heart phosphorylase is resolved chromatographically into three peaks (P_1, P_2, P_3). Peaks P_1 and P_2 are close to each other and can be separated only by slowly raising the concentration of glycerophosphate (first stage), but even then their separation is not complete. Peak P_3 separates well,

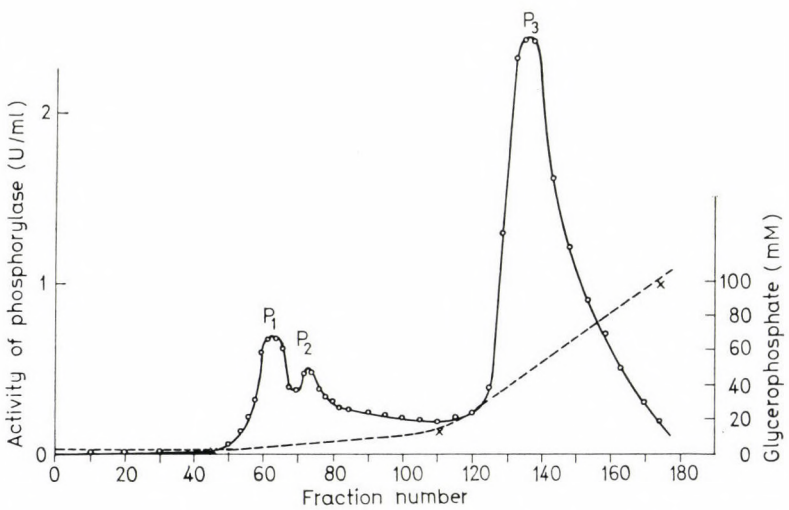

Fig. 1. Chromatogram of pig heart phosphorylase. 2200 units of purified heart phosphorylase (900 mg of protein) were chromatographed on a DEAE-cellulose column by two-step elu'ion with linear gradient. In step I (3—15 mM glycerophosphate) fractions 45 to 109, in step II (15—100 mM of glycerophosphate) fractions 110 to 175 were collected. The volume of each fraction was 15 ml, flow rate: 30 ml per hour

its elution in a small volume can be achieved by a steeper rise in glycerophosphate concentration (second stage).

Since the phosphorylase *b* activity of rabbit skeletal muscle can be greatly inhibited by G-6-P and ATP (Morgan, Parmeggiani, 1964b), an attempt was made to find some difference between the various pig heart isophosphorylases with respect to their inhibition by G-6-P and ATP.

The phosphorylase of pig skeletal muscle was compared with the easily eluted fraction P_1 of pig heart phosphorylase. Such a study was motivated by the chromatographic and immunological resemblance between the easily eluted fraction of rabbit heart isophosphorylases and the phosphorylase of rabbit skeletal muscle (Yunis et al., 1962). Since fraction P_1 of pig heart phosphorylase has properties greatly different from those of rabbit skeletal muscle phosphorylase, it can be assumed that pig skeletal muscle phosphorylase has equally different properties.

In order to be able to compare fraction P_1 of pig heart phosphorylase with the phosphorylase of pig skeletal muscle, first the properties of the latter had to be studied, since this type of phosphorylase is even less known and it markedly differs from rabbit skeletal muscle phosphorylase.

The inhibition by G-6-P and ATP of isophosphorylases obtained from pig heart and that of pig skeletal muscle phosphorylase are compared in Table 1.

Table 1 shows that of the heart phosphorylases the isoenzymes in fractions P_1 and P_2 can be inhibited only up to about 2 to 5% by G-6-P, while the inhibition of fraction P_3 is far more marked – 74%. It is further indicated that the phosphorylase of the pig skeletal muscle can be inhibited by G-6-P only to the same limited degree as fraction P_1 (or P_2) of the pig heart phosphorylase. Thus, similarly to rabbit phosphorylases, in the case of pig heart phosphorylases too the easily eluted fraction has properties which agree with those of the skeletal muscle

Table 1

The inhibitory effect of G-6-P and ATP on the activities of the skeletal muscle phosphorylase and of the heart isophosphorylases of pig

Composition of the incubation medium: 16 mM G-1-P, 1% glycogen, 10 mM Na-β-glycerophosphate, 0.5 mM EDTA, 7.5 mM mercaptoethanol, 1 mM AMP, pH 6.8, phosphorylase 0.25 unit per ml. Inhibition is expressed in percentage activity of the activity measured without inhibitor

Inhibitor mM	Inhibition, %			
	skeletal muscle phosphorylase	myocardiac phosphorylase fraction		
		P_1	P_2	P_3
G-6-P, 2	2	5	2	74
ATP, 8	37	40	40	20

phosphorylase. The phosphorylase in the pig skeletal muscle differs, however, greatly from the skeletal muscle phosphorylase of the rabbit with respect to its susceptibility to G-6-P and accordingly fraction P_1 of pig heart phosphorylase is also essentially different from fraction P_1 of rabbit heart phosphorylase.

Since phosphorylase *b* from rabbit muscle can be significantly inhibited by ATP too, a study of the effect of ATP on heart isophosphorylases (Table 1) seemed to be warranted. The results show that the activities of heart isophosphorylases differ with respect to their behaviour towards ATP, too. The activities of fractions P_1 and P_2 are inhibited by 8 mM ATP by about 40%, while the activity of fraction P_3 is inhibited only by about 20%. Thus, in contrast to the effect of G-6-P, ATP inhibits to a higher extent fraction P_1 and to a lesser extent fraction P_3. Also with respect to its inhibition by ATP, fraction P_1 behaved similarly to the skeletal muscle phosphorylase, since both were inhibited to about the same extent, about 37%, by ATP.

The different behaviour of isophosphorylases P_1 and P_3 towards G-6-P made it possible to determine directly the ratio between the two types of phosphorylases in the presence of an appropriate concentration of G-6-P. First the activities of the chromatographically separated fractions of the isophosphorylases, then the activity of the crude heart extract were investigated in the presence of increasing concentrations of G-6-P (Fig. 2).

Fig. 2 shows that the activity of fraction P_1 is not inhibited by G-6-P, not even when applied in a 20 mM concentration, while the activity of fraction P_3 is significantly reduced already by low concentrations of G-6-P and completely inhibited by 20 mM G-6-P.

On the basis of this experiment it could be assumed that in the crude heart extract even high concentrations of G-6-P are able to inhibit the activity of fraction P_3 only. The part of activity which cannot be inhibited may therefore be attributed to fractions P_1 and P_2. Fig. 2 indicates that in the crude extract contain-

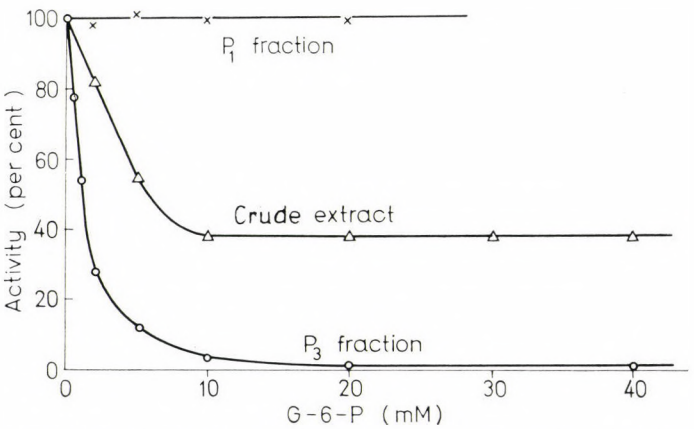

Fig. 2. The activities of chromatographically isolated heart isophosphorylases and of the phosphorylase in crude tissue extract in the presence of G-6-P. Enzyme activity was measured in the presence of 1 mM AMP

ing a mixture of isophosphorylases 10 mM G-6-P causes already maximum inhibition and a further increase in G-6-P concentration will cause no rise in the degree of inhibition. The residual part of activity which cannot be inhibited can be ascribed to the presence of fractions P_1 and P_2. The results indicate that in the crude extract the quantity of fraction P_3 which can be inhibited with G-6-P is about 60%, while fractions P_1 and P_2, which cannot be inhibited, are present in an amount of 40%. Similar results could be obtained by using the non-metabolizing 2-deoxy-G-6-P (Will, 1970).

The effect of AMP on isophosphorylases

The activity of rabbit phosphorylase *b* is known to be allosterically enhanced by AMP. The allosteric character is particularly pronounced in the presence of inhibitors (ATP or G-6-P) (Madsen, Shechosky, 1967; Wang et al., 1970). In the case of the phosphorylases P_1 and P_3 of the heart the allosteric interaction between the inhibitors and AMP is quite different. Fig. 3 shows the correlation between the activities of fractions P_1 and P_3 and AMP concentration with and without G-6-P.

Fig. 3 shows that the reaction rate does not change linearly with the concentration of AMP; the curve has a concave shape even without inhibitor, indicating that the changes in the activities of heart phosphorylases fail to follow Michaelis kinetics, similarly to the phosphorylase of the skeletal muscle of rabbit (Helmreich, Cori, 1964; Lowry et al., 1967). The concave reaction rate curve can be interpreted by assuming that it represents the homotropic cooperative inter-

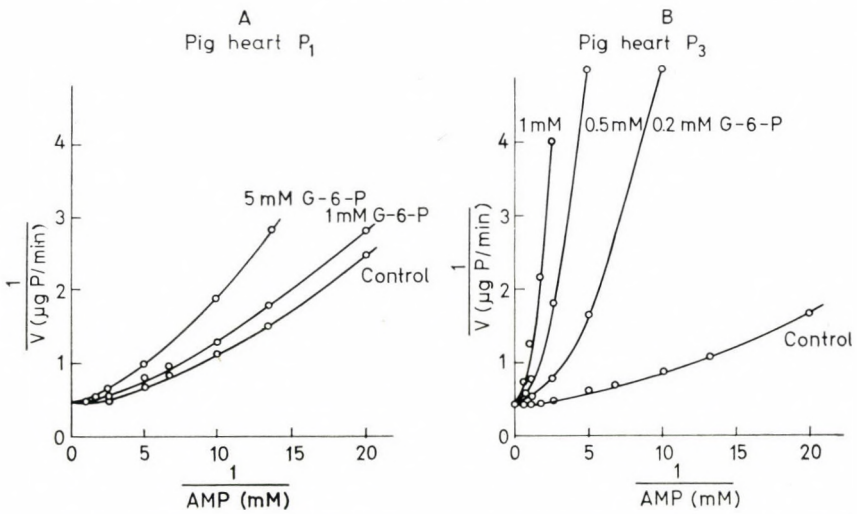

Fig. 3. The inhibitory effect of G-6-P on the activities of fractions P_1 and P_3 of pig heart phosphorylase *b*. A – Inhibition of fraction P_1 of pig heart phosphorylase by 1 mM and 5 mM of G-6-P. B – Inhibition of fraction P_3 of pig heart phosphorylase by 0.2 mM, 0.5 mM and 1 mM of G-6-P

action between the binding sites of phosphorylase *b* to AMP (Kastenschmidt et al., 1968; Wang et al., 1970).

In the presence of G-6-P the two isophosphorylases behave very differently. The activity of fraction P_1 (Fig. 3A) can be inhibited only at low AMP concentrations. Higher AMP concentrations suppress the inhibition. Since the value of V_{max} remains unchanged under the effect of G-6-P, the inhibitory action of G-6-P is of a competitive character with respect to AMP. In contrast to fraction P_1, fraction P_3 can be greatly inhibited by G-6-P and inhibition ceases only in the presence of high AMP concentrations (Fig. 3B). Since by raising the concentration of AMP the inhibition can be suppressed, the inhibition of fraction P_3 by G-6-P is with respect to AMP also competitive in nature. The results indicate that AMP influences the activities of fractions P_1 and P_3 to different degrees, and in the presence of G-6-P this difference is greatly enhanced. Since a double reciprocal plot of the results did not permit the determination of K_m (AMP), the results were also plotted according to Hill and the K_m (AMP) values of the two fractions were determined in this way (Fig. 4).

Fig. 4A shows the effect of AMP on the activity of fraction P_1. The K_m (AMP) value of the control (without G-6-P) is 1.1×10^{-4} M. In the presence of increasing concentrations of G-6-P the AMP activation curves are only very slightly shifted to the right, so that the K_m (AMP) values increase only slightly.

The value of the Hill coefficient which can be assigned to the control without G-6-P is $n = 1.66$ which agrees with the value for rabbit skeletal muscle phosphorylase *b* in the literature (Sealock, Graves, 1967; Lowry et al., 1967; Madsen, Shechosky, 1967). It can be seen that the *n* values of fraction P_1 do not change in the presence of G-6-P.

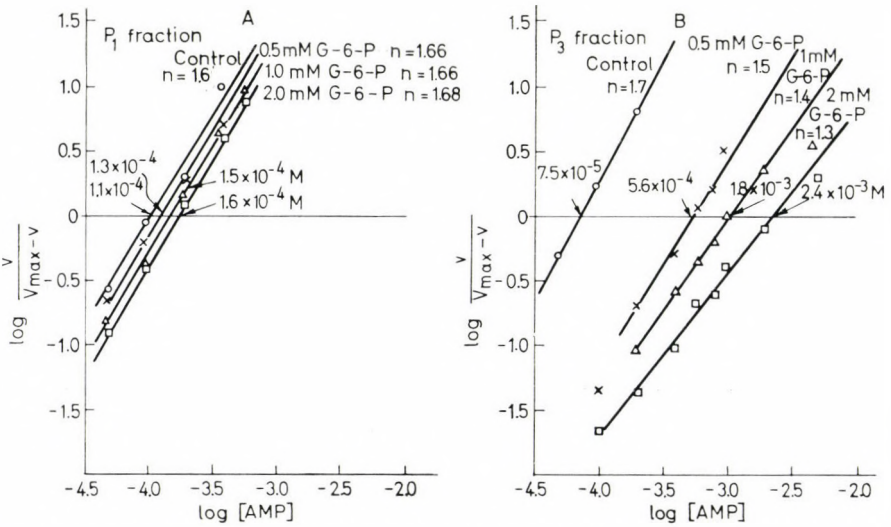

Fig. 4. Inhibitory effect of G-6-P on the isoenzymes of pig heart phosphorylase plotted according to Hill. A – Inhibition of fraction P_1. B – Inhibition of fraction P_3

Fig. 4B shows the AMP activation curves characteristic of fraction P_3 of heart phosphorylase both without G-6-P and in the presence of various concentrations of G-6-P. Without G-6-P the apparent K_m (AMP) value of 7.5×10^{-5} M is somewhat lower than the value observed for fraction P_1. In the presence of 0.5 mM, 1 mM and 2 mM of G-6-P the AMP activation curves are shifted considerably to the right, the pertaining apparent K_m (AMP) values are: 5.6×10^{-4}, 1.8×10^{-3} and 2.4×10^{-3} M, respectively. The increase in the K_m (AMP) values by several orders of magnitude indicates that G-6-P significantly reduces the affinity of the enzyme towards AMP. The Hill coefficient of fraction P_3 also changes in the presence of G-6-P. The value $n = 1.7$ without the inhibitor changes in the presence of 0.5 mM, 1 mM and 2 mM of G-6-P to $n = 1.5$, $n = 1.4$ and $n = 1.3$, respectively.

The inhibitory effect of ATP on the activities of fractions P_1 and P_3

Since ATP inhibits the activities of fractions P_1 and P_3 to different degrees (Table 1), it seemed worth investigating the nature of the interaction between AMP and ATP in the case of the two types of isophosphorylases. Fig. 5 shows the inhibitory effect of ATP on the activities of fractions P_1 and P_3 in the presence of increasing AMP concentrations.

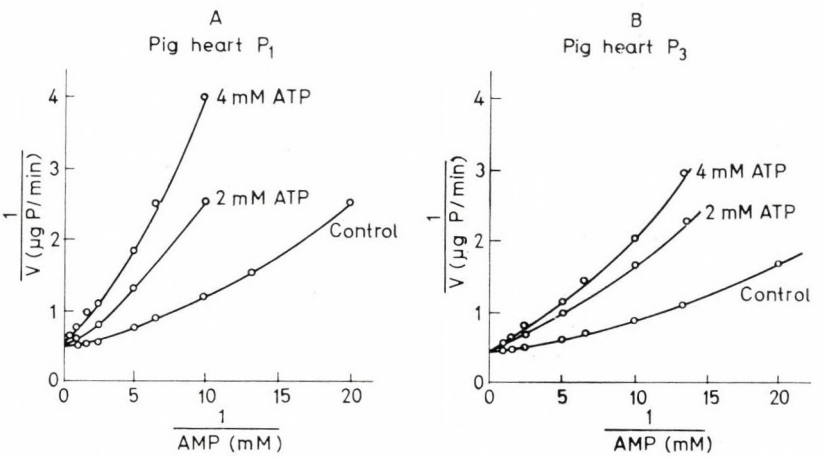

Fig. 5. Inhibitory effect of ATP on fractions P_1 and P_3 of pig heart phosphorylase. Concentrations of AMP and ATP as indicated

It can be seen that ATP significantly reduces the activities of both fractions; the activity of fraction P_1 is inhibited to a higher extent than that of fraction P_3. The inhibitory effect of ATP can be suppressed by raising the concentration of AMP; ATP does not alter the V_{max} of the enzymes. With both fractions P_1 and P_3 the shapes of the curves permit the conclusion that the inhibitory effect of ATP on AMP is of a competitive nature. Fig. 6 shows the results plotted according to Hill's method.

Fig. 6A shows that as a result of the effect of ATP (4 mM and 8 mM, respectively) the curve of fraction P_1 is considerably shifted to the right and the apparent K_m (AMP) increases from 1.2×10^{-4} M to about its fivefold, to 5.9×10^{-4} M and to about its ninefold, to 11×10^{-4} M, respectively. The decrease of the Hill coefficients as a result of the presence of ATP indicate presumably a reduction in the cooperative interaction between the binding sites for AMP.

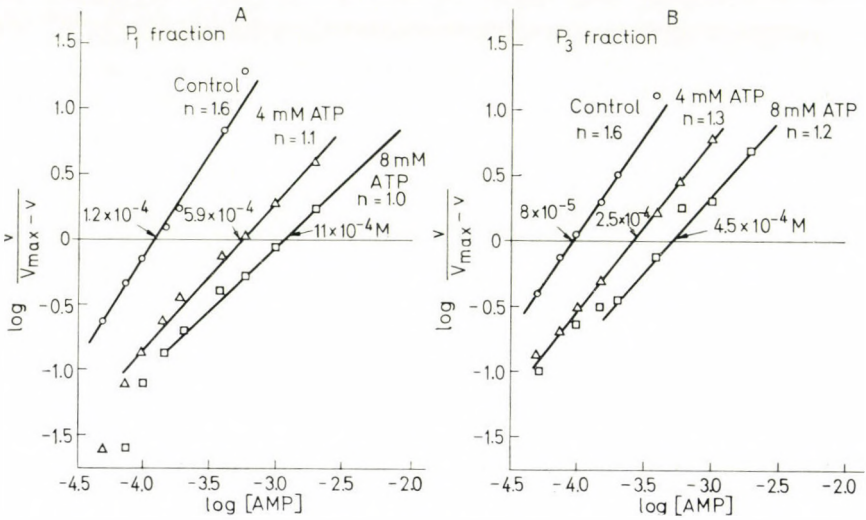

Fig. 6. Inhibitory effect of ATP on the isoenzymes of pig heart phosphorylase plotted according to Hill. A – ATP inhibition of fraction P_1. B – ATP inhibition of fraction P_3

In case of fraction P_3 (Fig. 6B) the apparent K_m (AMP) value also increases under the effect of ATP. 4 mM ATP raises the K_m value to about three times, 8 mM ATP to about five times the initial value, thus to a lesser degree than in the case of fraction P_1. In the case of fraction P_3 too the Hill coefficients decrease slightly.

Thus ATP has an inhibitory effect on both fractions of pig heart phosphorylase; it reduces somewhat more markedly the affinity of fraction P_1 for AMP than that of fraction P_3.

Discussion

Our investigations have shown that the isophosphorylases of the pig heart are also allosteric enzymes, similarly to the much investigated phosphorylase of the skeletal muscle of rabbit. This has been confirmed by the non-linear shape of the activation curves if represented on a double reciprocal plot and by the fact that the slopes of the Hill plots were higher than one.

According to our results the isophosphorylases of the pig heart represent two types which differ also with respect to their allosteric properties. Fraction P_1

is closely related to the skeletal muscle phosphorylase not only with respect to its immunological and chromatographic properties (Yunis et al., 1962), but also as far as its allosteric properties are concerned. Similarly to pig skeletal muscle phosphorylase fraction P_1 of the pig heart phosphorylase cannot be inhibited by G-6-P, in contrast to the phosphorylase of the skeletal muscle of rabbit. Our results are in agreement with the findings of Hanabusa and Kohno (1969) and of Will et al. (1970).

In contrast to fraction P_1, fraction P_3 of the pig heart phosphorylase is characterized by a marked sensitivity for G-6-P. The enzyme can be inhibited to a high extent also by low concentrations of G-6-P and G-6-P also greatly reduces the affinity of fraction P_3 for AMP.

Fraction P_2 is generally considered the hybrid of fractions P_1 and P_3. The properties of fraction P_2 of pig heart phosphorylase are primarily determined by the properties of fraction P_1, which can only partly be attributed to the eventual presence of small amounts of fraction P_1 as an impurity in fraction P_2.

The inhibitory effect of ATP on the isophosphorylases of the pig heart is; similarly to phosphorylase *b* of rabbit skeletal muscle, of a competitive nature with respect to AMP; the apparent K_m (AMP) values and the Hill coefficients both change.

ATP inhibits fractions P_1 and P_3 to a different extent: the activity of fraction P_1 is more inhibited than that of fraction P_3. Fraction P_3 which can be greatly inhibited by G-6-P is less sensitive to ATP, while fraction P_1 which almost completely resists inhibition by G-6-P is more sensitive to ATP.

The strong inhibitory effect of G-6-P on fraction P_3 seems to indicate that the mechanism of G-6-P inhibition is not the same as that of ATP inhibition, despite the fact that both have an inhibitory effect of competitive nature with respect to AMP. The difference in the character of the two types of inhibition is confirmed by recent tests showing that the inhibitory action of G-6-P is only partly competitive with that of AMP (Wang et al., 1970).

Since the concentrations of G-6-P, AMP and ATP in the heart are sufficiently high to influence the activity of phosphorylase *b* (Neely et al., 1968), it may be assumed that also in vivo these effectors will regulate the activity of heart phosphorylases and thereby the glycogenolysis in the heart. However, in this regulation the different isophosphorylases may have different roles.

Due to the fact that in anoxia the ATP and G-6-P concentrations of the heart drop, while AMP concentration rises, it may be assumed that phosphorylase has a catalytic activity even in form *b*. Fraction P_3 is the one which may primarily participate in this catalytic activity, since it can be better regulated than fraction P_1. The function of fraction P_3 is inhibited to a lesser degree by ATP which is an important fact, since in the initial stage of anoxia the ATP concentration of the heart shows almost no decrease (Krause, Wollenberger, 1965), so that at this stage fraction P_3 may catalyze glycogenolysis with better effect. On the other hand the high sensitivity of fraction P_3 to G-6-P ensures the regulating effect of G-6-P and can thereby hinder the complete depletion of tissue glycogen.

In contrast to fraction P_3 fraction P_1 of heart phosphorylase is practically not inhibited by G-6-P, so that the activity of fraction P_1 remains unaffected even after G-6-P accumulation and its effect is regulated only by the ratio ATP/AMP.

According to the above the isophosphorylases of the heart permit a differentiated control mechanism which ensures the regulation of the glycogenolysis of the heart even under anoxic conditions.

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Maleylation of Myosin

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The effect of maleylation on the enzymic and physicochemical properties of myosin was studied. The following observations were made.

1. The reaction with maleic anhydride causes a decrease in the number of both amino and sulfhydryl groups of myosin. The decrease is much less pronounced if maleylation is conducted in the presence of β-mercaptoethanol.

2. Both Mg^{2+} and Ca^{2+}-mediated ATPase activities of myosin yield maximum curves as a function of maleylation if β-mercaptoethanol is present during the treatment, whereas in its absence only the Mg^{2+}-mediated ATPase activity shows a maximum curve. The K^{+}-activated ATPase activity of myosin monotonously decreases as a result of maleylation under either condition.

3. Maleylation changes both the intrinsic viscosity and the α-helix content of myosin as compared with the untreated samples.

Introduction

The introduction of negatively charged groups by the reaction of dicarbonic acid anhydrides with lysyl residues is extensively used for the modification of proteins. The effect of succinylation on the molecular parameters and characteristic properties of myosin is known from the studies of several authors (Oppenheimer et al., 1966, 1967; Bárány, Oppenheimer, 1967). In the present paper the changes in the biological and physicochemical properties of myosin caused by the reaction with maleic anhydride are reported.

Maleic anhydride was introduced into protein chemistry as an amino group reagent by Butler et al. (1967). With protein not containing SH groups it could be shown (Butler et al., 1967, 1969; Uyeda, 1969) that maleic anhydride specifically reacts with the lysyl residues. This reagent has been used in some recent studies on the primary structure (Freedman et al., 1968) and active groups (Uyeda, 1969) of proteins.

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Materials and methods

Myosin was prepared essentially as described by Portzehl et al. (1950). It was purified further by centrifuging at 105 000 $\times g$ in a Spinco L 50 preparative ultracentrifuge for 1 hour. Myosin was precipitated 4 times to remove low molecular weight protein contaminants before the measurement of intrinsic viscosity. Fresh myosin, not older than 7 days, was used throughout. All the chemicals used were of reagent grade.

Protein content was measured by the biuret method of Gornall et al. (1949).

Maleylation was carried out at 0 °C by using a stock solution of 3 M maleic anhydride in absolute ethanol, from which different quantities were added to a 5 mg/ml (10^{-5} M) myosin solution, in 0.5 M KCl, 0.1 M borate buffer (pH 8 or 9) and in some experiments 10 mM β-mercaptoethanol. The stock solution of maleic anhydride was prepared immediately before use. During the reaction the pH was kept constant by neutralizing with 1 M KOH in a Radiometer pH-stat (TTT1/SBR2). After 10 minutes no more alkali was consumed. The samples were left standing overnight when all traces of maleic anhydride hydrolyzed to maleic acid. The samples were then dialyzed against 100 volumes of 0.5 M KCl and 30 mM borate buffer at the pH of the test solution to remove maleic acid, ethanol and β-mercaptoethanol. The temperature was kept at 0 °C throughout.

ATPase activity was measured in a solution containing 1 mg/ml of myosin, 4 mM ATP, 0.5 M KCl, 20 mM borate buffer (pH 8), and alternatively, 5 mM EDTA or 5 mM $CaCl_2$ or 2 mM $MgCl_2$. The measurements were carried out with 2 ml samples at 20 °C. The incubation was terminated by the addition of 2 ml of 10% trichloroacetic acid. P_i was measured by the method of Fiske and SubbaRow (1925). The incubation time was chosen so as to obtain a decomposition of ATP less than 25%. The ATPase activity was expressed as μmole P_i per mg of myosin per min.

The absorption spectra were measured in a Spektromom 202 spectrophotometer.

Amino groups were determined essentially as described by Habeeb (1966): 1 ml of 4% $NaHCO_3$ and 1 ml of 0.2% trinitrobenzenesulfonic acid were added to 1 ml of myosin solution (0.15 mg per ml in 0.5 M KCl + 20 mM borate buffer, pH 8). The samples were incubated at 40 °C for 2 hours in the dark and were dialyzed against 30 volumes of distilled water to remove the excess of reagent. The absorbance of the dialysed samples was measured at 346 nm. The number of amino groups was estimated from the measured absorbance difference by using the extinction coefficient given by Okuyama and Satake (1960): $\Delta\varepsilon_{346} = 1.45 \times 10^4$. The number of sulfhydryl groups was determined by the method of Ellman (1959) in the presence of 8 M urea. The intrinsic viscosity was measured in an Ubbelohde viscometer on samples of 0.5–0.1 mg/ml of myosin, at 20 °C. The samples were diluted with 0.5 M KCl + 20 mM borate buffer solution (pH 8 or 9).

The molecular weight of myosin was taken to be 500 000 daltons.

Results

It was shown by Butler et al. (1967, 1969) and Freedman et al. (1968) that maleic anhydride specifically reacts with the amino groups of proteins. Since these authors worked with proteins not containing free sulfhydryl groups, it seemed of interest to study the effect of maleylation on myosin, which contains beside the amino-groups also cysteinyl residues. Maleylation of ε-NH_2 groups was evaluated by the above investigators from the spectral differences observed in the ultraviolet region. This spectrophotometric method proved to be unsuitable in the case of myosin owing to the changes in turbidity, which made it impossible to make a quantitative estimation of maleylated lysyl residues from the measured absorption spectra.

Myosin was maleylated in both the presence and absence of β-mercaptoethanol at pH 8 or 9. The numbers of both free NH_2 and SH groups decrease on maleylation as shown in Table 1. The reaction of both groups is faster at higher pH. At pH 8 the percentual decrease in the NH_2 groups as a function of added maleic anhydride is higher than that in SH-groups, whereas, at pH 9 the decrease in the number of SH groups is higher than that of NH_2 groups. The presence of 10 mM β-mercaptoethanol markedly protects both groups from maleylation at either of the above pH values.

The ATPase activity of myosin samples maleylated under different conditions was measured by adding alternatively 5 mM Ca^{2+}, 5 mM EDTA or 2 mM Mg^{2+}. The Ca^{2+} activated ATPase activity as a function of maleic anhydride concentration (Fig. 1) exhibits a maximum curve with a high maximum at either pH if

Table 1

Reaction of amino and sulfhydryl groups of myosin with maleic anhydride

For determination of amino and sulfhydryl groups and for the details of maleic anhydride treatment see Methods. Untreated myosin contains 370 amino groups and 43 sulfhydryl groups per molecule

β-mercapto-ethanol	Maleic anhydride concentration $M \times 10^2$	Maleic anhydride to myosin molar ratio	Number of groups, reacted, moles/mole of myosin			
			pH 8		pH 9	
			$-NH_2$	$-SH$	$-NH_2$	$-SH$
absent	0.1	100	41.4	3.4	57.3	15.0
	0.3	300	83.6	5.2	86.2	27.7
	1.0	1000	179.0	6.8	219.0	35.4
	5.0	5000	334.1	31.1	363.3	39.7
present	0.1	100	14.8	1.7	20.3	4.4
	0.3	300	30.3	0.0	30.3	5.5
	1.0	1000	35.1	2.1	38.8	5.0
	5.0	5000	79.9	31.8	113.6	37.4

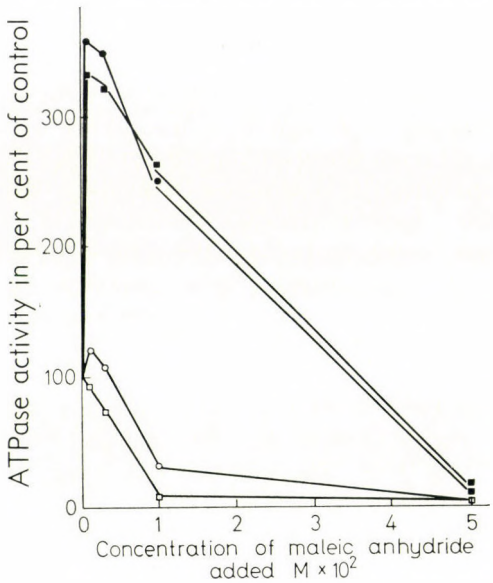

Fig. 1. Effect of maleylation on the Ca^{2+}-activated ATPase activity of myosin. ATPase activity was measured in the presence of 5 mM $CaCl_2$. Maleylation was carried out in the presence (●, ■) or in the absence (○, □) of β-mercaptoethanol at pH 8 (○, ●) or at pH 9 (□, ■). Control (100%) activity: 0.243 μmoles P_i per mg myosin per min both in the presence and absence of β-mercaptoethanol

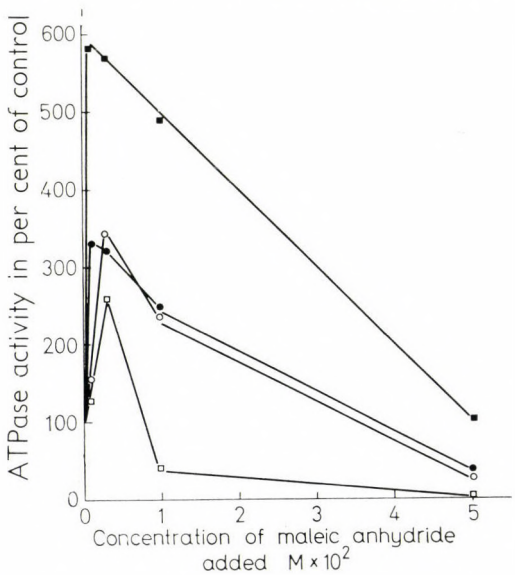

Fig. 2. Effect of maleylation on the Mg^{2+}-mediated ATPase activity of myosin. ATPase activity was measured in the presence of 2 mM $MgCl_2$. Maleylation was carried out in the presence (●, ■) or in the absence (○, □) of β-mercaptoethanol at pH 8 (○, ●) or at pH 9 (□, ■). Control (100%) activity: 0.0058 μmoles P_i per mg myosin per min both in the presence and absence of β-mercaptoethanol

β-mercaptoethanol was present during maleylation. In the absenc eof β-mercaptoethanol Ca^{2+}-ATPase activity sharply decreases with increasing maleic anhydride concentration at pH 9 and shows a scanty maximum at pH 8.

The Mg^{2+}-mediated ATPase activity of myosin as a function of maleic anhydride concentration (Fig. 2) markedly increases with increasing concentrations of the reagent at both pH 8 and 9. Especially high activation (580%) was measured with myosin maleylated in the presence of β-mercaptoethanol at pH 9.

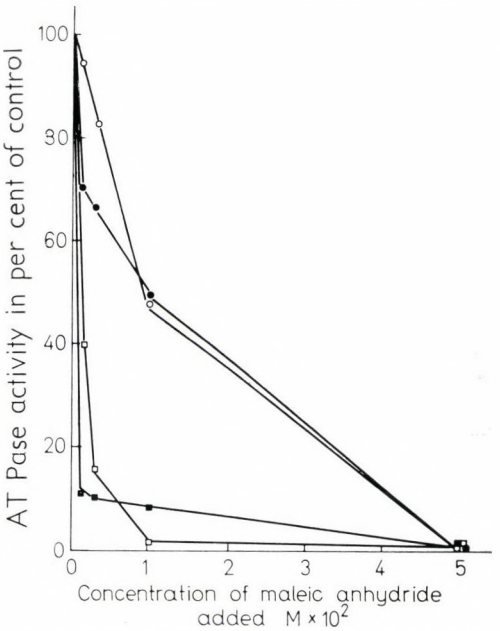

Fig. 3. Effect of maleylation on the K^+-activated ATPase activity of myosin. ATPase activity was measured in the presence of 5 mM EDTA. Maleylation was carried out in the presence (●, ■) or in the absence (○, □) of β-mercaptoethanol at pH 8 (○, ●) or at pH 9 (□, ■). Control (100%) activity: 0.93 μmoles P_i per mg myosin per min both in the presence and absence of β-mercaptoethanol

The EDTA-activated ATPase activity of myosin, which is equivalent to the K^+-activated ATPase activity (Mühlrad et al., 1964), decreases with increasing maleic anhydride concentration irrespective of the conditions of maleylation (Fig. 3). The decrease is particularly sharp at pH 9.

The effects of maleylation on the intrinsic viscosity and helicity of myosin were also studied. The change of intrinsic viscosity with increasing maleylation si shown in Fig. 4. The intrinsic viscosity of myosin increased with increasing maleylation. Viscosity was affected by low maleic anhydride concentrations if no β-mercaptoethanol was present. A maximum curve was obtained at pH 9 in the absence of β-mercaptoethanol: at the highest maleic anhydride concentration used the intrinsic viscosity gave the value measured with the untreated

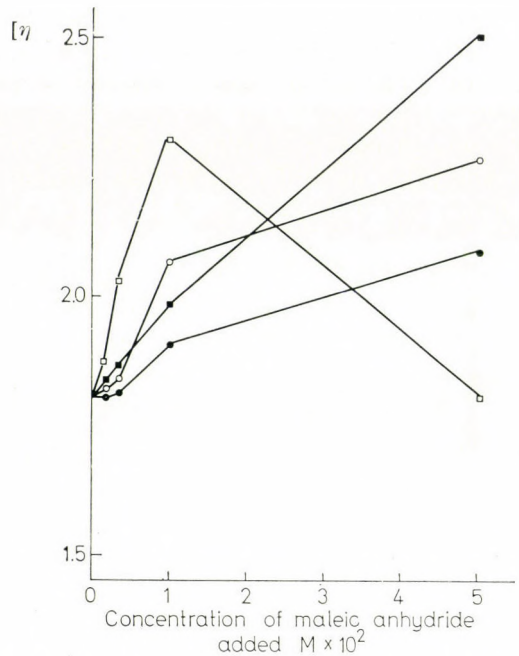

Fig. 4. Effect of maleylation on the intrinsic viscosity of myosin. For the details of viscosity measurement see Methods. Maleylation was carried out in the presence (●, ■) or in the absence (○, □) of β-mercaptoethanol at pH 8 (●, ○) or at pH 9 (■, □)

Table 2

Effect of maleylation on the α-helix content of myosin

Optical rotatory dispersion measurements were carried out in the range from 270 to 235 nm in an Opton REP spectropolarimeter. Helicity was calculated by the method of Simmons et al. (1966) from the rotation at 233 nm. Conditions of the determination: 0.5 mg/ml of myosin, maleylation was performed at pH 8

Maleic anhydride concentration M × 10²	Molar ratio of maleic anhydride to myosin	Helicity (%) 10 mM-mercaptoethanol	
		absent	present
—	—	55.2	55.5
0.1	100	48.4	47.3
0.3	300	51.1	51.5
1.0	1000	44.6	46.8
5.0	5000	21.6	42.2

control. A similar curve was obtained by Oppenheimer et al. (1967) on the succinylation of myosin.

The α-helix content of myosin decreases appreciably only at a high degree of maleylation, as shown by Table 2. β-mercaptoethanol does not completely

prevent this effect of maleylation but the α-helix content of myosin treated with 5000 moles of maleic anhydride per mole of myosin in the presence of β-mercaptoethanol was found to be twice as high as that of myosin maleylated with the same concentration of the reagent but without β-mercaptoethanol.

Discussion

It has been shown in the present experiments that maleic anhydride reacts specifically not only with the amino groups of the proteins but also with their free SH groups (Table 1). A decrease in the number of measurable SH groups was also observed by Oppenheimer et al. (1967) and by Mühlrad et al. (1968) on the succinylation of SH-containing proteins. Attempts at the demonstration of S-acyl derivatives produced by the maleylation of myosin (unpublished measurements) remained unsuccessful. These derivatives are probably very unstable under the experimental conditions used (Riordan, Vallee, 1964).

When maleylation is performed in the presence of β-mercaptoethanol the decrease in the number of amino groups, as well as in that of the sulfhydryl groups, is much less pronounced. The protection from maleylation of the functional groups can be attributed only partly to the direct reaction between β-mercaptoethanol and maleic anhydride, which necessarily reduces the amount of maleic anhydride available for reaction with the functional groups of myosin. If this direct reaction with maleic anhydride were the only cause of decreased maleylation, this could be overcome by higher concentrations of maleic anhydride. However, the Ca^{2+}-activated ATPase activity of myosin is enhanced by maleylation in the presence of β-mercaptoethanol to such a high extent which is never observed at low maleic anhydride concentrations in the absence of this reagent. Similarly, the K^{+}-activated ATPase activity decreases more rapidly as a function of the concentration of maleic anhydride added in the presence of β-mercaptoethanol than in its absence. These observations suggest that the amino and sulfhydryl groups reacting with maleic anhydride in the presence and absence of β-mercaptoethanol are not the same.

Oppenheimer et al. (1967) observed the complete disappearance of the Ca^{2+}-activated ATPase activity of myosin succinylated with 500 moles of reagent per mole of myosin. In the case of maleylation, however, an appreciable Ca^{2+}-activated ATPase activity of myosin was observed even at 1000 : 1 maleic anhydride to myosin molar ratio.

The effects of modifications of the sulfhydryl or amino groups on the ATPase activity of myosin have been already thoroughly studied. The blocking of SH groups usually results in the activation of both Ca^{2+} and Mg^{2+}-mediated ATPase activities (Kielley, Bradley, 1956; Levy, Ryan, 1961; Sekine, Kielley, 1964; Morales, Hotta, 1960; Perry, Cetterill, 1965), whereas modification of the amino groups causes only the Mg^{2+}-mediated ATPase activity to increase (Kubo et al., 1960; Kitagawa et al., 1961; Tokuyama, Tonomura, 1967; Fábián, Mühlrad, 1968). In the case of maleylation, however, it could not be decided yet whether

the observed changes in the enzymic properties are brought about by the blocking of amino or sulfhydryl groups, since both Mg^{2+}- and Ca^{2+}-mediated ATPase activities are enhanced by maleylation in the presence of β-mercaptoethanol, whereas in the absence of this thiol compound only Mg^{2+}-activated ATPase activity was found to be enhanced.

The introduction of a large number of negative charges causes a substantial change in the shape and structure of the myosin molecule as inferred from the changes of intrinsic viscosity and the helicity of maleylated myosins. The changes are similar to those observed by Oppenheimer et al. (1967) on the succinylation of myosin.

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Tracer Kinetic Analysis of Phosphate Incorporation into Erythrocytes in vitro

I. A Simple Model for Simultaneous Investigation of Phosphate Transport and Exchange in Erythrocytes

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The process of incorporation of labeled phosphate into red blood cells has been studied in vitro. To describe exactly the process of incorporation and to study simultaneously the two possible types of incorporation, exchange and transport, tracer kinetic model analysis has been applied. Four pools (intracellular and extracellular inorganic phosphate, and intracellular organic acid-labile and acid-resistant phosphate) of acid-soluble phosphate compounds were isolated and their amounts and activities determined as functions of time. The system can be described as a closed model with four compartments. To choose a model which is isomorphous with our system, experimental data have been analyzed with a digital computer according to all of the logically possible model variations. The isomorphous model arrived at enables the simultaneous description of exchange and transport with information on the rate of metabolism of acid-soluble phosphate compounds.

Introduction

In erythrocytes inorganic phosphate (P_i) is transferred across the membrane by passive transport (Dunker, Passow, 1953; Pfleger, 1960; Vestergaard-Bogind, 1963; Gerlach et al., 1964; Deuticke, 1967). However, there are observations suggesting the simultaneous existence of another transfer mechanism. The data of several authors suggest an indirect exchange between ATP-phosphorus of erythrocytes and extracellular P_i at the membrane (Gourley, 1952; Bartlett, 1958; Schauer, Hillmann, 1961; Latzkovits et al., 1966). This latter process might be relevant at low extracellular P_i concentration when the passive transport does not operate (Gourley, 1952; Bartlett, 1958; Latzkovits et al., 1966). Thus in tracer kinetic analysis of P_i transport it is very important to distinguish between two possible types of P_i incorporation: one is the transport associated with a change in concentration of extracellular P_i and the other is the exchange taking place at constant concentration. The latter can be kinetically represented as two opposite transport processes with the same velocity. In the tracer kinetic description, the case is further complicated by the fact that the incorporated P_i plays a prominent role in the metabolism as well. Therefore the metabolic processes should be included in the tracer analysis of P_i transport and exchange.

Investigations in which the rates of P_i transport and exchange were simultaneously determined and interrelations between transport and metabolism were

taken into account have not been presented in the literature. Several authors have tried to determine the rate of P_i influx and efflux in different experiments using resealed ghosts (Glader, Omachi, 1968; Schrier, 1970). However, influx and efflux could not be determined under the same experimental conditions and consequently, although they yield very important information about particular problems, experiments with resealed ghosts cannot substitute for tracer kinetic treatment.

The purpose of the present investigations was to elaborate a tracer kinetic compartmental model to allow a simultaneous study of P_i transport, exchange and metabolism *in vitro*. The basic concepts of tracer kinetic analysis using compartmental models are outlined in the works of Brownell et al. (1968), Solomon (1960) and Branson (1961). The terms used in our paper correspond to those proposed by Brownell et al. (1968) for the nomenclature of tracer kinetics.

Although the metabolism of red blood cells is simple compared to that of other cells, a large number of phosphate compounds and several metabolic relations between them must be assumed. Therefore if all the phosphate compounds in red blood cells were regarded as separate pools, and accordingly, as independent compartments in the tracer kinetic model, a mathematical description would be rather difficult. But the principle of model construction as defined by Solomon (1960), i.e. the use of the possibly smallest number of compartments when developing a model suitable for the description of a given system, contradicts the application of such a detailed compartment structure. Accordingly, in our experiments only the following pools of acid-soluble phosphate compounds were regarded as independent compartments:

A: Extracellular P_i
B: Intracellular P_i
C: Intracellular organic acid-labile phosphate
D: Intracellular organic acid-resistant phosphate

These four pools were chosen for the following reasons: 1) Our experiments indicate that the labeling of the acid-insoluble compounds is negligible as compared to that of the acid-soluble compounds. 2) The P_i in the system is separated by the membrane into an extracellular and an intracellular compartment. 3) Organic compounds belonging to the acid-labile pool are of the same type with respect to the stability of phosphate bonds, representing the "high energy pool". 4) The acid-resistant pool consists of compounds of the same type with respect to the stability of phosphate bonds and their role played in phosphate metabolism.

In the course of our experiments we tried to prove that such chemically heterogeneous compartments, relatively few in number, are suitable for inclusion in a simple model by which P_i transport and exchange as well as P_i metabolism could be simultaneously investigated *in vitro*.

Materials and methods

The experiments were carried out with red blood cells of adult, healthy men and women. Blood, with citrate as anticoagulant, was centrifuged immediately after sampling at $0 - +1°$, 800–1000 rpm, for about 15–20 min. Plasma and

buffy coat were removed and the cells were washed three times with 3–5 volumes of cold physiological saline. Washed cells were suspended in 4 volumes of Krebs–Ringer phosphate solution of the following composition:

Na^+	0.118 M
K^+	0.0047 M
Mg^{2+}	0.0025 M
Ca^{2+}	0.0011 M
$Na_2HPO_4 - NaH_2PO_4$	0.012 M
Glucose	100 mg%
pH	7.35
Radioactive concentration	1 μCi ^{32}P/ml

The suspension was incubated at 37° with gentle shaking, and samples were taken at 15, 30, 45, 60, 90, 150 and 180 min. After rapid cooling, the samples were centrifuged at 0 – +1°.

The cell-free supernatant was separated and inorganic phosphate was isolated and determined as P-molybdate in benzene–isobutanol at 0°, according to the somewhat modified Martin–Doty method (Martin, Doty, 1949; Pfleger, Seifen, 1962). The cells of the suspension were washed 3 times with 20 volumes of ice-cold physiological saline and precipitated with 4 volumes of 12.5% trichloroacetic acid (TCA). After centrifuging, inorganic phosphate was isolated from the TCA supernatant and measured as described above. An aliquot was used for spectrophotometric determinations and another for radioactivity measurements.

Another sample of the TCA supernatant was hydrolyzed in 1 N sulphuric acid for 10 min at 100°. Inorganic phosphate was then isolated and measured by the modified Martin–Doty method (Martin, Doty, 1949; Pfleger, Seifen, 1962). The difference between the values of hydrolyzed and nonhydrolyzed samples gives the concentration and activity of the so-called acid-labile fraction.

Determination of the phosphate content and of total activity of TCA supernatants was carried out after wet combustion by a method developed in our Institute (Domonkos, Heiner, 1968). In some cases wet combustion of whole red blood cells was carried out to measure total incorporation and to check whether the labeling of the acid-insoluble fraction is negligible.

The radioactivity of benzene–isobutanol phases was measured with a Packard Tri-Carb spectrometer, type 3375, in a toluene–alcoholic medium with POPOP and PPO scintillators. The activity of the aqueous solutions obtained by wet combustion of TCA supernatants was also measured in the same spectrometer by the Cherenkov effect.

Since the different fractions were analyzed by the same method as was inorganic phosphate, the quenching effect was disregarded and results are given as cpm. Relative efficiencies of the Cherenkov and scintillation measurements were determined and used as correction factors. The determination of this relative efficiency has been done by internal standardization.

Concentrations, total activities and specific activities of extracellular inorganic (A), intracellular inorganic (B), intracellular acid-labile (C) and intracellular acid-resistant (D) phosphate will be shown as functions of time.

The ^{32}P-labeled phosphate used was a carrier-free, sterile product of the Isotope Institute of the Hungarian Academy of Sciences.

Mathematical treatment

To describe the kinetics of the tracer distribution in the closed compartment system defined by the four pools, A–D (and with A regarded as the precursor), the formalism of Branson (1961) has been applied. Considering all logically possible interconnections of the four compartments and assuming that the steady state is disturbed only by the passive P_i transport from compartment A to B, we can set up the following equations:

$$\frac{dI_A}{dt} - \frac{dA}{dt} S_A = W_{AC}(S_C - S_A) + W_{AB}(S_B - S_A) + W_{AD}(S_D - S_A) \quad (1)$$

$$\frac{dI_B}{dt} + \frac{dA}{dt} S_A = W_{AB}(S_A - S_B) + W_{BC}(S_C - S_B) + W_{BD}(S_D - S_B) \quad (2)$$

$$\frac{dI_C}{dt} = W_{AC}(S_A - S_C) + W_{BC}(S_B - S_C) + W_{CD}(S_D - S_C) \quad (3)$$

$$\frac{dI_D}{dt} = W_{AD}(S_A - S_D) + W_{BD}(S_B - S_D) + W_{CD}(S_C - S_D) \quad (4)$$

where I_A, I_B, I_C and I_D are total activities of pools A, B, C and D, respectively (cpm per ml washed blood cell or per 4 ml Krebs–Ringer solution); S_A, S_B, S_C and S_D are specific activities of the four pools, A – D (cpm per μg phosphorus); W_{XY} is the rate of exchange between the two pools denoted by the indices (μg phosphorus per min per ml washed blood cell or 4 ml Krebs–Ringer solution) (by definition the values of W_{XY} must be positive or zero (Brownell et al., 1968; Solomon, 1960; Branson, 1961)); and A, B, C and D are the amounts of phosphate of the particular pools (μg phosphorus per ml washed blood cell or 4 ml Krebs–Ringer solution).

It can be seen that the incorporation determined by the net transport from A to B is given as $(dA/dt)S_A$ in equations (1) and (2). Therefore, by assuming that transport is the only process disturbing the steady state in the system, interactions of the four pools can be treated in the kinetic description as exchange processes. Similar equations can be found in the work of Reiner (1953a, 1953b) and in the book of Neiman and Gál (1971). Thus in the tracer kinetic treatment, the two possible types of P_i incorporation are separately determined as a consequence of the general formalism.

In equations (1)–(4), six exchange rates are involved, representing the logically possible interconnections. Considering that any one of these exchange rates

may in fact be zero as well, we can define a set of models, each representing a given scheme of interconnection of the four compartments. The equations describing the models can be deduced from equations (1)–(4) by substituting zero for the negligible exchange rates. The number of all of these models can be given as follows:

$$\binom{6}{6} + \binom{6}{5} + \binom{6}{4} + \binom{6}{3} + \binom{6}{2} + \binom{6}{1} = 63$$

It is to be expected that only some of the models, perhaps only one of them, should be satisfied by experimental data (Brownell et al., 1968). These models (or model) are (is) considered as isomorphous to the system. The numerical values of the differential quotients were obtained with the same accuracy by either numerical or graphical differentiation of the curves.

After excluding the models clearly contradicting experimental data, a digital computer (type Minsk 22) was used to solve the equations. In determining the numerical values of exchange rates, we did not assume that they were constant. However, in the case of models involving more than three exchange rates (only three of equations (1)–(4) are independent), the equations could be solved only by setting them up for pairs of near points of time and supposing the exchange rates to be constant within short intervals.

Results

First it was determined whether the labeling of phosphate compounds other than acid-soluble ones need be taken into consideration. It is known that certain phosphoproteins and phospholipids can be rapidly labeled. Therefore, changes in total activity of red blood cells during incubation were compared with activity increase of TCA supernatants. Table 1 shows the means of these results as a func-

Table 1

Differences in total activities of acid-soluble phosphates and the whole cell

Time of incubation, min.	$\left[1 - \frac{I_{AS}}{I_{RBC}}\right] \times 100$
0	−0.33
15	−0.82
30	0.06
45	−1.10
60	0.68
90	0.91
120	0.15
150	0.80

I_{AS}: Total activity of acid soluble phosphates in 1 ml of washed red blood cells. (Mean values of 70 experiments.)

I_{RBC}: Total activity of 1 ml of washed red blood cells. (Mean values of 70 experiments.)

tion of time, from ten experiments, the total activity of the red blood cell taken as 100%.

From data of Table 1, it can be stated that the labeling of phosphate compounds other than acid-soluble ones can be neglected with a good approximation when compared with acid-soluble total activity. Accordingly, in our *in vitro* experiments the acid-soluble pool can be regarded as a closed system.

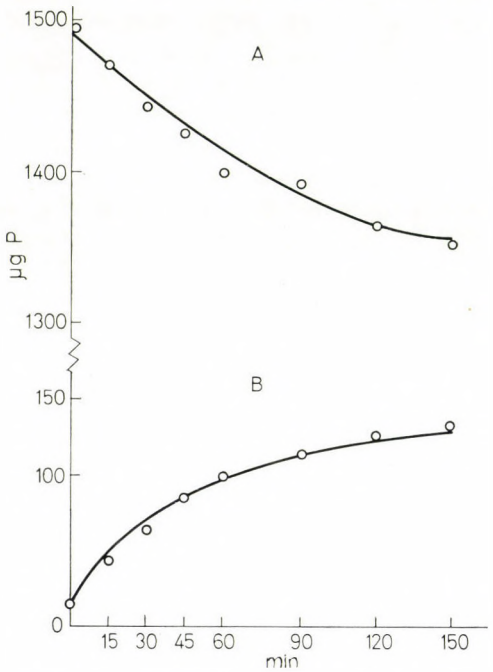

Fig. 1. Change in the amount of the extra- and intracellular P_i in 4 ml of Krebs–Ringer solution and 1 ml of washed red blood cells, respectively, versus time. A: Extracellular P_i, B: Intracellular P_i. Each point of the curves represents the mean value of 70 experiments. Standard deviations are within 7.5%

Fig. 1 demonstrates the change in pools A and B, and Table 2 shows the mean values for C and D. To describe the transport, a curve-fitting procedure was performed by using the digital computer, and the following function was obtained:

$$-\frac{dA}{dt} = 1.9e^{-0.02t} \tag{5}$$

In 80 experiments, the amount, total activity and specific activity of the A – D pools were examined as functions of time. In about 90% of experiments, the steady state condition is fulfilled for compartments C and D. The amount of pool A decreased continuously, and simultaneously the amount of pool B increased to the same extent owing to the P_i transport from A to B.

Table 2

Changes of acid-labile and acid-resistant phosphates (pools C and D) in 1 ml of washed red blood cells during incubation

Time of incubation, min.	Pool D	Pool C
0	310 ± 20*	91 ± 8.0*
15	305 ± 21	89 ± 6.5
30	312 ± 17	95 ± 7.8
45	315 ± 22	90 ± 9.1
60	300 ± 19	88 ± 7.5
90	320 ± 22	93 ± 8.8
120	312 ± 20	97 ± 9.0
150	303 ± 16	89 ± 8.3

* Mean values in μg P/ml washed red blood cells and ± Standard deviations of 70 experiments.

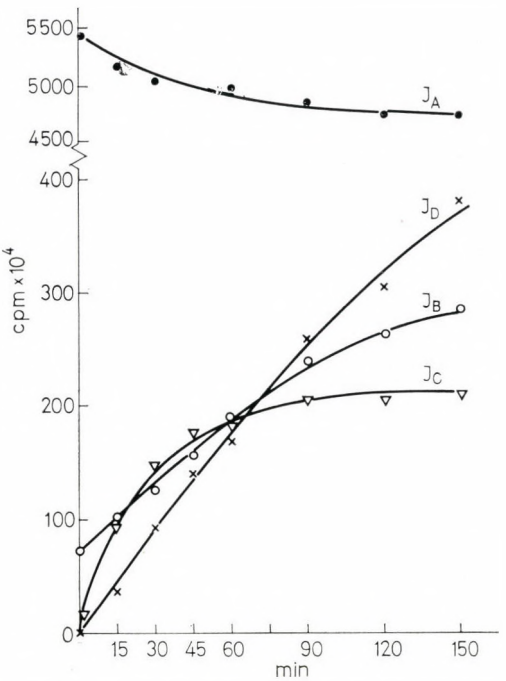

Fig. 2. Change in total activity of the phosphate pools in 1 ml of washed red blood cells and in 4 ml of Krebs—Ringer solution, respectively, versus time. I_A: extracellular P_i pool, I_B: intracellular P_i pool, I_C: acid-labile phosphate pool, I_D: acid-resistant phosphate pool. Each point of the curves represents the mean value of 70 experiments. Standard deviations are within 7.5%

Changes in total and specific activities are demonstrated in Figs 2 and 3, respectively. It is conspicuous that the curves of I_B and S_B start with definite value at zero time, and the curve of S_C intersects the S_B curve.

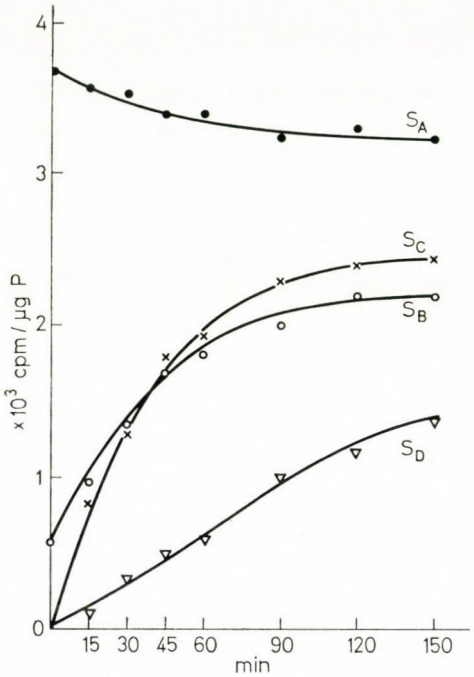

Fig. 3. Specific activity curves of the phosphate pools. S_A: extracellular P_i, S_B: intracellular P_i, S_C: acid-labile phosphate, S_D: acid-resistant phosphate. Each point of the curves represents the mean value of 70 experiments. Standard deviations are within 7.5%

A detailed study of experimental data expressed in Figs 1–3 permits the reduction of the number of possible models. In Fig. 3, curve S_C still increases markedly when:

$$S_C > S_B > S_D$$

Since in this case the specific activity of pool A is greater than that of pool C, S_C can further increase only if the $A \rightleftarrows C$ exchange exists in the system. Thus the isomorphous model should include exchange $A \rightleftarrows C$.

Setting up Eq. (2) with the experimental data for a point in time when S_C is greater than S_B, we can see that the left side of the equation

$$\frac{dI_B}{dt} + \frac{dA}{dt} S_A$$

is less than zero, whereas the first two terms on the right side

$$W_{AB}(S_A - S_B) + W_{BC}(S_C - S_B)$$

cannot be negative. Hence, the equation can be satisfied only if the third term

$$W_{BD}(S_D - S_B)$$

has a negative value. That means that W_{BD} must be greater than zero and, consequently, exchange $B \rightleftarrows D$ exists. Thus the isomorphous model should include exchange $B \rightleftarrows D$, too.

If we sum Eq. (1) and Eq. (3) the following equation is obtained:

$$\frac{dI_A}{dt} - \frac{dA}{dt} S_A + \frac{dI_C}{dt} = W_{AB}(S_B - S_A) + W_{AD}(S_D - S_A) + W_{BC}(S_B - S_C) +$$

$$+ W_{CD}(S_D - S_C)$$

By setting up this equation with the experimental data for a point in time when S_B is greater than S_C, the left side of the equation turns out to be positive. At the same time, the first, second and fourth terms of the right side cannot be positive. Hence, the third term

$$W_{BC}(S_B - S_C)$$

must be positive. That means that W_{BC} must be greater than zero and, consequently, exchange $B \rightleftarrows C$ exists.

Thus we can establish that the isomorphous model should certainly include the following three exchanges:

$$A \rightleftarrows C, \qquad B \rightleftarrows C, \qquad B \rightleftarrows D$$

By establishing these exchanges, the number of possible models turns out to be

Table 3

Structures of the logically possible 8 models

Models	Possible interconnections					
	$A \rightleftarrows B$	$A \rightleftarrows C$	$A \rightleftarrows D$	$B \rightleftarrows C$	$B \rightleftarrows D$	$C \rightleftarrows D$
1	O	X	O	X	X	O
2	X	X	O	X	X	O
3	O	X	X	X	X	O
4	X	X	X	X	X	O
5	O	X	O	X	X	X
6	X	X	O	X	X	X
7	O	X	X	X	X	X
8	X	X	X	X	X	X

X: exchange exists,
O: exchange does not exist.
Each of the models involves the P_i transport across the membrane.

rather limited and can be given as follows:

$$1 + \binom{3}{1} + \binom{3}{2} + \binom{3}{3} = 8$$

The schemes of these models are demonstrated in Table 3. Equations (1)–(4) were set up for each of these eight models with the experimental data and solved for exchange rates. Seven of the eight models provided negative exchange rates as is shown in Table 4. Accordingly, we can establish that these seven models

Table 4

Negative exchange rate values of W_{BD} *in the contradictory models (μg P/min/ml washed red blood cells)*

Time of incubation, min.	Models						
	2	3	4	5	6	7	8
0	− 3.61	−0.286	− 2.800	− 0.432	− 1.921	−0.10	-3.10^7
15	− 0.653	−1.303	− 0.760	−11.00	− 1.581	−1.15	-1.10^7
30	− 0.139	−7.102	−0.550	− 7.379	− 0.371	−0.34	-7.10^7
45	−58.591	−3.312	−2.875	−10.186	− 0.83	−0.67	-5.10^7
60	− 6.397	−3.186	−2.049	−13.239	− 0.347	−1.31	-2.10^7
90	− 9.168	−3.120	−3.929	− 7.190	−10.699	−0.96	-1.10^7
120	− 0.65	−4.886	−0.452	− 6.660	−26.937	−0.01	-1.10^7
150	− 0.300	−5.250	−0.580	− 5.250	−30.250	—	-9.10^6

Rate values were calculated from the kinetic curves in Figs 1, 2 and 3.

cannot be satisfied by experimental data and must be rejected. Thus we can give the only model which is satisfied by the experimental data. This isomorphous model is shown in Fig. 4. The numerical values of the exchange rates in the isomorphous model are demonstrated in Table 5. The isomorphism of this model in the investigated system is supported by the fact that the exchange rates are constant with good approximation.

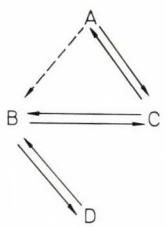

Fig. 4. Scheme of the isomorphous model. Solid lines: exchanges, dotted line: P transport across the membrane

Table 5

Exchange rate values of the isomorphous model shown in Fig. 4 (μg P/min/ml washed red blood cells)

Time of incubation, min.	W_{AC}	W_{BC}	W_{BD}
0	1.27	1.31	3.8
15	1.20	2.94	2.45
30	1.20	2.13	2.29
45	1.25	7.47	2.65
60	1.23	6.62	2.61
90	1.26	6.97	2.69
120	1.20	7.55	2.62
150	1.02	6.26	1.56

Rate values were calculated from the kinetic curves in Figs 1, 2 and 3.

Discussion

Brownell et al. (1968) have stressed that "A danger in the use of compartmental models is the tendency to interpret the elements of the model directly in terms of system elements without adequate confirmation". In connection with our model, demonstrated in Fig. 4, this danger is striking. It is evident that further detailed information is necessary to make clear the meaning of the three exchange interconnections included in the model in relation to the metabolism of erythrocytes. Nevertheless, in view of the practical significance of models in general (Locker, 1968; Bertalanffy, 1950), our model is of value. The practical advantages of this model are as follows: 1) The numerical values of the exchange taking place simultaneously with the transport across the membrane are given by the values of W_{AC}. 2) By numerical values of W_{BC} and W_{BD}, the metabolism of the organic phosphate compounds within the cell can be represented. 3) The numerical rate values are constant with good approximation. 4) It follows from the foregoing that $^{32}P_i$ tracer experiments can be described by applying our model.

Considering that about 80% of the C pool, as is well known, consists of ATP (Hinsberg, Lang, 1951), the existence of the $A \rightleftarrows C$ exchange is in good agreement with published data suggesting the incorporation of P_i directly into ATP at the membrane (Gourley, 1952; Bartlett, 1958; Schauer, Hillmann, 1961; Latzkovits et al., 1966). At the same time our findings suggest that the kinetics of P_i transport from compartment A to B, expressed in Eq. (5), agree with the data in the literature, which indicate passive P_i transport (Dunker, Passow, 1953; Pfleger, 1960; Gerlach et al., 1964; Vestergaard-Bogind, 1963; Deuticke, 1967). Thus the model enables us to reconcile somewhat contrasting observations concerning P_i incorporation, which were earlier regarded as inevitably conflicting.

It is rather conspicuous that curves I_B and S_B, in Figs 2–3, start with finite initial values instead of zero. Such a value for the initial activity of the intracellular pool indicates a process taking place at low temperature in the first 1–2 min after mixing washed red blood cells and labeled Krebs–Ringer phosphate solution. From the initial value of intracellular inorganic total activity and extracellular specific activity the amount of phosphate transported by this process can be calculated; in our experiments it is 1–2 μg phosphorus per ml washed red blood cell. This value does not increase if, even before the start of the reaction, the cells are in contact with the Krebs–Ringer phosphate solution for a longer time at low temperature. This observation indicates that the above-mentioned rapid process is in equilibrium already at the start of the experiment. This is the reason why this process need not be considered when constructing an isomorphous model. However, this finite, initial value of the S_B curve could be the cause that the values of W_{BC} (see Table 5) are not constant in the first 30–45 min.

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Effect of Cysteine on Haematopoietic Tissues

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Four days after a single intraperitoneal injection of 1000 mg/kg L-cysteine-HCl · H_2O, the number of spleen colony-forming units (CFU) was reduced in the femoral marrow of BALB/c mice. The number of CFU in spleen was decreased two days after the administration of cysteine, and showed an overshoot on the fourth day.

Incorporation of thymidine-2-^{14}C into bone marrow DNA in vivo was reduced when administered 30 minutes, 6 hours, 4, 8 or 10 days after cysteine.

These alterations in haematopoietic tissues were not accompanied by changes in the radioresistance of the whole mice ($LD_{50/30}$ of ^{60}Co gamma rays) except 30 minutes after the administration of cysteine.

Our finding that the incorporation of labeled thymidine into bone marrow DNA is inhibited by cysteine administered 30 minutes earlier is interpreted as an inhibitory effect of cysteine on cells in the S-phase. This delay in DNA synthesis may facilitate DNA repair and, thus contribute to the radioprotection afforded by cysteine to the whole animal.

Introduction

Mortality of mice induced by midlethal exposure to ionizing radiation is due primarily to damages of the haematopoietic tissues, mainly of the haematopoietic stem cell population (Bond et al., 1965). Protection of the stem cell population from radiation damage, therefore, may play an important role in chemical radioprotection. It has been established that some radioprotectors, among them cysteine, really protect spleen colony-forming units, i.e. haematopoietic stem cells (Smith et al., 1966; Duplan, Fuhrer, 1966; Ainsworth, Larsen, 1969; Jurásková, Tkadlecek, 1967; Tkadlecek, Jurásková, 1967; Fehér et al., 1968; Kovács et al., 1971). Earlier experiments of ours (Kovács et al., 1971; Kovács, Hernádi, 1971) suggested that cysteine, in addition to the aforementioned radioprotective effect, is likely to disturb the "normal" steady-state relationships in haematopoietic tissues. This disturbance seemed to merit further investigation, since it had been proposed that the metabolic alterations induced by sulfur-containing substances explain the radioprotective effect of these compounds (Bacq, Goutier, 1967). In the present paper the effects of cysteine on haematopoetic tissues are described and an attempt is made to correlate these effects with the radioprotection afforded by cysteine to the whole animal.

Materials and methods

Animals

Estimation of $LD_{50/30}$ of ^{60}Co gamma ray was carried out on 12 to 18-week-old male and female BALB/c mice bred in a closed colony. The other experiments were performed on 10 to 16-week-old inbred BALB/c mice of both sexes. Mice were kept on a standard laboratory diet and water *ad libitum*. Bone marrow and spleen-cell recipient mice were kept five to a cage and their drinking water was acidified to pH 3.0 with hydrochloric acid.

Irradiation procedure

Whole body irradiation of mice was performed with a ^{60}Co gamma source at an exposure rate of 56 R/min. During irradiation mice were kept in a plexiglass box.

Cysteine administration

L-cysteine-HCl · H_2O was administered as a single intraperitoneal dose of 1000 mg per kg of body weight in a volume of 0.2 ml/10 g body weight. The freshly prepared solution of cysteine was adjusted to pH 6.0 with sodium hydroxide.

Estimation of the number of colony-forming units in femoral marrow and spleen

The effect of cysteine on the population of the spleen colony-forming units was studied with the method of Till and McCulloch (1961) as follows:

At various times after the administration of cysteine, animals were killed by cervical dislocation in groups of five. Single cell suspensions were prepared from pooled marrow of the ten femurs and from the five pooled spleens in ice-cold Puck's saline G solution (Puck et al., 1958). The number of bone marrow and spleen cells was determined by standard laboratory methods. After appropriate dilution, suitable quantities of the bone marrow and spleen cell suspensions were injected within 2 hours after killing the animals (Carsten, Bond, 1968) into the lateral tail vein of recipient mice irradiated previously with 720 R of ^{60}Co gamma rays. On the 9th day following transplantation, the mice were killed, spleens were fixed in Bouin's fluid and the macroscopically visible colonies on the surface of the spleens were counted.

In preliminary experiments, an exposure to 720 R of ^{60}Co gamma rays proved to be suitable for irradiating recipient mice prior to grafting, because the number of endogenous spleen colonies arising after this exposure was sufficiently low (0.385 ± 0.23; mean $\pm$ SE, 26 mice). To obtain the net number of exogenous colonies per recipient spleen, the value of 0.385 colonies per spleen was subtracted from the mean number of colonies found in the spleens of grafted animals. The values of colony-forming units (CFU) per femur, CFU per 10^5 nucleated bone marrow cells and CFU per 10^6 nucleated spleen cells were cal-

culated from the actual number of net exogenous colonies found in the spleens of recipients. No corrections were made for the fraction of CFU which had actually lodged in spleen and for the extinction probability of colonies (Matioli et al., 1970).

Incorporation of thymidine-2-^{14}C into bone marrow DNA

At various times after the administration of cysteine, male BALB/c mice were given intravenously 1 μCi thymidine-2-^{14}C per 20 g body weight. Sixty minutes later the mice were killed and the femoral marrow was washed out and extracted twice with ice-cold 0.5 N perchloric acid. The samples were then incubated in 0.5 N perchloric acid at 90° for 25 minutes. After centrifugation, the DNA content of the supernatant was determined according to Burton (1956). The ^{14}C-radioactivity of the same supernatants was measured in a liquid scintillation spectrometer (Type NZ-137, Gamma, Budapest) with the liquid scintillant containing Triton-X-100 as recommended for 0.5 N perchloric acid by Fox (1968). Samples were counted to less than 5% counting error.

Determination of the 30-day LD_{50} of ^{60}Co gamma rays

Groups of mice were exposed to different doses of ^{60}Co gamma rays. $LD_{50/30}$ values and the 95% confidence limits were calculated from 30-day mortality data according to Litchfield and Wilcoxon (1949).

Chemicals

Chemicals were obtained from the following sources:
L-cysteine-HCl · H_2O, toluene, reagent grade, Reanal, Budapest;
Thymidine-2-^{14}C, specific radioactivity 58 mCi per mole, Philips Duphar;
PPO, scintillation grade, Nuclear Enterprises, Edinburgh;
POPOP, Nuclear Chicago;
Triton-X-100, pract., Serva, Heidelberg.

Results

The number of the nucleated bone marrow cells per femur after the administration of cysteine did not differ, beyond experimental variations, from the control value (1.86×10^7, Fig. 1, lower curve).

The number of CFU in the femoral marrow of untreated animals was 1506 ± 133 CFU per femur or 7.99 ± 0.68 CFU per 10^5 nucleated bone marrow cells (mean ± SE; 3 independent experiments with 5 donor mice each, a total of 22 recipients). The number of CFU per femur did not change during the first two days after the administration of cysteine. From the 4th day on lower values were found. However, the decrease of the number of CFU proved to be statistically significant only on the 4th and 10th day (Fig. 1, middle curve).

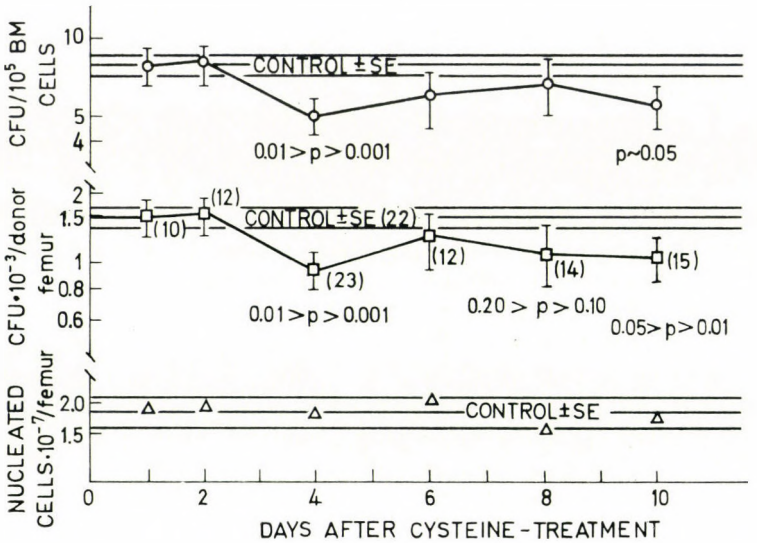

Fig. 1. Number of nucleated bone marrow cells per femur (lower curve), exogenous colon-y forming units (CFU) per femur (middle curve) and CFU per 10^5 nucleated bone marrow cells (upper curve) plotted against time after the intraperitoneal administration of 1000 mg per kg body weight L-cysteine-HCl · H_2O. Vertical bars indicate standard error; number of recipient spleens are shown in parentheses. Values of p were calculated by Student's method

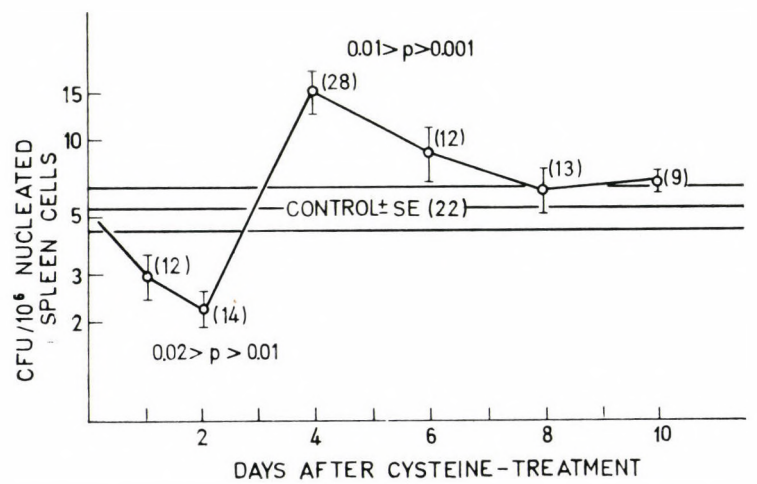

Fig. 2. Number of exogenous colony-forming units per 10^6 nucleated spleen cells plotted against time after the intraperitoneal administration of 1000 mg per kg body weight L-cysteine-HCl · H_2O. Vertical bars indicate standard error; number of recipient spleens are shown in parentheses. Values of p were calculated by Student's method

Similar results were obtained by expressing the number of net exogenous colonies relative to the number of nucleated bone marrow cells injected into the recipients. In this way the reduction in the number of CFU was found to be at the limit of statistical significance on the 10th day after cysteine-treatment ($p \sim 0.05$, Fig. 1, upper curve).

The number of CFU per 10^6 nucleated spleen cells was also influenced by cysteine treatment, as shown in Fig. 2.

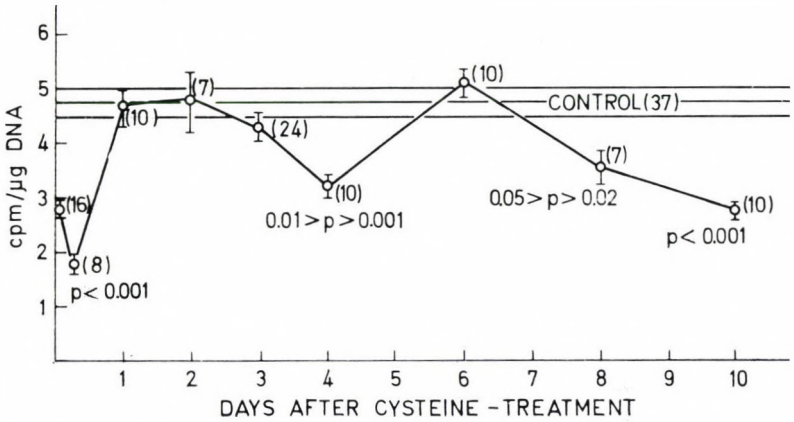

Fig. 3. Specific radioactivity of bone marrow DNA 60 minutes after the intravenous injection of 1 μCi per 20 g body weight thymidine-2-^{14}C plotted against the time elapsed between the intraperitoneal administration of 1000 mg per kg body weight L-cysteine-HCl · H_2O and that of the labeled thymidine. Vertical bars indicate standard error; number of femurs are shown in parentheses. Values of p were calculated by Student's method

This value can be taken to be approximately proportional to the absolute number of CFU per spleen, since no remarkable deviations from the control were observed in the average number of nucleated cells per spleen in cysteine-treated mice (control: 2.2×10^8 cells per spleen). In comparison with a control value of 5.46 ± 0.98 CFU per 10^6 nucleated spleen cells, a statistically significant decrease was observed on the second day after the administration of cysteine. On the 4th day, however, a statistically significant increase was found followed by a gradual return by the 8th day to a level slightly above the control.

The specific radioactivity of bone marrow DNA measured 60 minutes after the injection of thymidine-2-^{14}C is plotted against the time elapsed between the administration of cysteine and that of labeled thymidine in Fig. 3. This value is supposed to be proportional to the rate of DNA synthesis (Nygaard, Potter, 1959), i.e. to the overall rate of cellular proliferation. As can be seen from Fig. 3, the incorporation of labeled thymidine was decreased when injected 30 minutes or 6 hours after cysteine but returned to the control in 24 hours. A second decrease was observed on the 4th day and a third one on days 8 to 10.

According to the above results, cysteine influences the population size of the haematopoietic stem cells (CFU) both in bone marrow and spleen, as well as the overall rate of cellular proliferation in bone marrow.

To see whether these alterations of the haematopoietic tissues are accompanied by any changes in the radiation resistance of mice, the $LD_{50/30}$ ray of ^{60}Co gamma rays was estimated on mice irradiated at various times following cysteine administration. Table 1 shows that, except for an increase in the first hour after the administration of cysteine (which was already described by Bacq, 1965), the $LD_{50/30}$ did not change over the whole period of observation.

Discussion

It has been suggested in our earlier papers that cysteine disturbs the "normal" steady-state relationship in haematopoietic tissues (Kovács et al., 1971; Kovács, Hernádi, 1971). Our present data support the above suggestion.

As CFU exhibit several properties which may be considered characteristic of normal haematopoietic stem cells (Till, McCulloch, 1967), the changes in the number of CFU both in femoral marrow and spleen after the administration of cysteine (Figs 1 and 2) may be interpreted as changes in the size of stem cell population.

Several authors have described phenomena indicating that some chemical radioprotectors, even in unirradiated animals, affect the function of lymphohaemopoietic organs. Injection of mercaptoethylamine reduces the peripheral lymphocyte count and increases the granulocyte count in mice (Smith et al., 1965). According to Braun (1968) and Braun and Albach (1970), cysteine reduces the weight of thymus in mice by inducing the migration of lymphocytes from the thymic cortex and stimulates the reticulum in the same organ.

It is also known that the incorporation of labeled precursors into DNA of various organs is substantially reduced by certain chemical radioprotectors (Bacq, Goutier, 1967; Brasch, 1969; Brasch, Fehér, 1969) such as mercaptoethylamine, cysteine and aminoethyl-isothiouronium bromide.

Changes in the incorporation of labeled precursors into DNA observed after any kind of treatment may indicate that the process of DNA synthesis is affected by the treatment. However, as it has been explained in detail by Lajtha (1960), some other possibilities, such as population changes, population shifts, cell cycle changes, and pool changes, should also be considered. The decrease in the incorporation of ^{14}C-thymidine, injected 30 minutes after the administration of cysteine (Fig. 3), can hardly be attributed to changes either in cell-population size or composition, or in precursor pools. Therefore, this phenomenon is most likely the result of the inhibitory action of cysteine on cells in S-phase. Six hours after the administration of cysteine, G_1 inhibition and/or population shift also may contribute to the observed decrease in thymidine incorporation. The fall in thymidine uptake 4 and 8 to 10 days after treatment with cysteine may be due to population shift as also suggested by the observed changes in the number of CFU (Fig. 1), although the possibility of alterations in pool size cannot be excluded.

Changes in the number of CFU or in the incorporation of labeled thymidine into bone marrow DNA were not accompanied by alterations in the radioresistance

Table 1

Radiation resistance ($LD_{50/30}$) of non-inbred BALB/c mice after the intraperitoneal administration of 1000 mg per kg body weight L-cysteine-HCl · H_2O

Time after the administration of cysteine	Number of animals	$LD_{50/30}$ *R*	95% confidence limit
Control (no cysteine)	86	806.4	757.1—858.8
30 min	44	885.2	790.3—991.5
2 days	71	798.5	746.2—854.4
4 days	30	794.5	746.6—845.4
8 days	34	826.0	737.6—882.0

of (whole) mice (Table 1) except 30 minutes after the administration of cysteine. This finding is in agreement with the view of Ainsworth and Larsen (1969), who claim that a close correlation between the number of CFU and radioresistance of the whole animal exists only if "normal" steady-state relationships prevail in the haematopoietic tissues. As pointed out by Braun and Albach (1970), the cysteine-induced reduction of the weight of the thymus cannot be correlated with the radioprotective effect of cysteine since the same effect is produced by the non-protecting isocysteine too.

However, our finding that the incorporation of labeled thymidine into bone marrow DNA is inhibited by cysteine administered 30 minutes before, is consistent with the view of Bacq and Goutier (1967). According to their hypothesis, a delay in DNA synthesis or mitosis induced by sulphur-containing radioprotectors increases the efficacy of the (DNA) repair system.

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Iodination of Penicillinase Isoenzymes

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Penicillinases produced by inducible and constitutive mutants of B. cereus strain 569 have been shown to represent each a mixture of isoenzymes. Both purified enzyme preparations consist of three isoenzymes, two of them showing similar and one dissimilar electrophoretic mobility. Each penicillinase isoenzyme in itself reacts with iodine in two independent steps as shown earlier for the mixtures of isoenzymes. When treated with iodine at pH 9, each isoenzyme incorporates iodine (2 atoms/1 molecule enzyme). The present experiments support the view that the inactivation of penicillinase by iodine is the result of two independent reactions and the partial inactivation of the enzyme takes place by partial inactivation of each individual molecule.

Introduction

Evidence that the inactivation of penicillinase by iodine takes place in two independent steps has been presented earlier (Csányi et al., 1970). Eliciting conformational change by a powerful agent it was demonstrated that the two independent reactions were not related to two enzyme conformations of dissimilar iodine sensitivity (Mile et al., 1970). Imsande et al. (1970) separated the penicillinase produced by the inducible mutant of B. cereus 569 into 3 isoenzymes by acrylamide gel electrophoresis. To clarify whether or not the two steps of iodine inactivation were due to an eventual difference in the iodine sensitivities of the isoenzymes, the iodine reaction of each isoenzyme was examined in detail in these laboratories.

Materials and methods

Penicillinases produced by B. cereus 569 and B. cereus 569/H and purified by a method described earlier (Csányi et al., 1970) were used throughout the experiments.

Disc electrophoresis of penicillinase

Disc electrophoresis of penicillinase was carried out in the following way: The container buffer was a mixture of 0.05 M Tris and 0.38 M glycine, adjusted to pH 8.5. For electrophoresis glass tubes 120 mm long and 6 mm in

diameter were used. The gel was placed in the tubes up to a height of 73 mm. The gel containing 7% Cyanogum 41 (acrylamide+N,N'methylenebisacrylamide; American Cyanamide Co.) was prepared as described by Imsande et al. (1970). After allowing to stand for 2–3 hours, ammonium persulfate was removed from the gels by preliminary electrophoresis at 10–12 V/cm for 3 hours, using Tris buffer, pH 8.9, as solvent, at the same concentration as present in the gel. Twelve tubes were run simultaneously. After preliminary electrophoresis 0.05–0.1 ml of penicillinase solution (30–120 μg penicillinase dissolved in 40% saccharose) was applied to the gel surface in each tube. Subsequently container buffer was cautiously layered over the gels and electrophoresis was started in the refrigerator at 2–8°C and 3–5 mA current intensity per tube. Each container contained 1 liter of buffer solution. The samples were run for 2.5–4 hours. Part of the gels was stained by 0.5% amido black. For enzyme- or radioactivity assay the gel pieces were cut into discs 1 mm thick. For radioactivity assay the discs were placed directly in the dioxane-containing "cocktail" and scintillation was counted with a Tri-carb spectrometer. Prior to enzyme assay the discs were eluted each in 1 ml solution containing 0.25 M NaCl + 0.05 M phosphate, pH 6.5, for 10–12 hours.

Iodination of penicillinase with ^{131}I

1–3 mg penicillinase was treated in a volume of 1 ml with 10^{-3} M iodine + 3×10^{-3} M KI which contained 10–30 μCi ^{131}I. Iodination was carried out at 0°C for 1 minute. Excess iodine was removed by 10^{-2} N thiosulfate solution, KI was added in a tenfold excess over the iodine content of the material and iodide was removed by gel filtration, using a Sephadex G-25 column, 5 by 600 mm in size. It was ascertained in control experiments that under the above conditions no measurable amount of radioactive iodide ions was bound by the enzyme. The method used to measure the activity of penicillinase was described earlier (Csányi, 1961).

Results

Penicillinase isoenzymes of the constitutive and inducible mutants of B. cereus 569

Imsande et al. (1970) have shown that the penicillinase produced by the inducible mutant of B. cereus consisted of 3 isoenzymes. In these laboratories the finding of Imsande et al. was reproduced by means of disc electrophoresis (Fig. 1) and, in addition, the penicillinase produced by the constitutive mutant B. cereus 569/H was shown to be separable into 3 isoenzymes as well, by the same method. The electrophoretic behaviour of the isoenzymes of the two strains was not fully uniform. As shown in Fig. 2, the constitutive enzyme had one slower migrating fraction as compared to the inducible enzymes, whereas the inducible enzyme had, in contrast, one faster migrating fraction as compared to the constitutive enzymes. Two fractions in each enzyme had identical electrophoretic mobilities. Thus, simultaneous running of the two enzyme preparations resulted in 4 electrophoretic fractions.

Imsande et al. (1970) have examined whether treatment with concentrated urea would change the separation of inducible penicillinase into fractions of dissimilar mobilities. The fact that they failed to demonstrate such a change was one proof that the fractions have been isoenzymes rather than artefacts.

To obtain further proof of the isoenzyme nature of the fractions, the constitutive enzyme was treated with 0.1 N NaOH for 5 minutes at 0°C and after acidifying to pH 6, immediately subjected to electrophoresis. As can be seen from Fig. 3

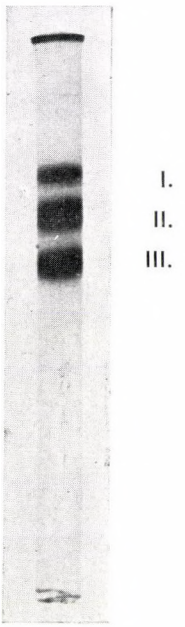

Fig. 1. Electrophoresis of constitutive exopenicillinase. 100 μg penicillinase was separated electrophoretically for 3 hours as described in the text and the electrophoretogram was stained

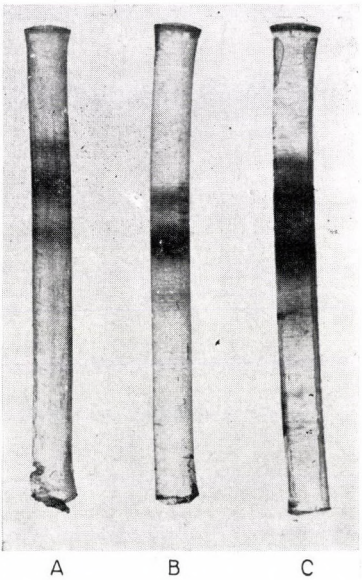

Fig. 2. Electrophoretograms of exopenicillinase produced by constitutive and inducible mutants of B. cereus 569. A, 80 μg exopenicillinase from constitutive mutant; B, 80 μg exopenicillinase from inducible mutant; C, 40 μg constitutive + 40 μg inducible enzyme; run for 3.5 hours and stained

even this very drastic treatment failed to affect either the mobilities or the quantitative relations of the fractions. It has been previously shown (Mile et al., 1970) that treatment with alkali under the above conditions had no notable influence on enzyme activity, and that the change in conformation caused by the alkali treatment was reversible.

Two of the three constitutive isoenzyme fractions could be separated for preparative purposes. The method of separation is described below.

The preparations containing the isoenzymes were applied to Celite 545 adsorbent and eluted as described previously (Csányi et al., 1970). Celite 535, used for the preparation of the enzyme, did not change the proportions of the three

isoenzymes, whereas Celite 545, recently applied for chromatographic separation, diminished the quantity of the isoenzyme intermediate between the two others with respect to electrophoretic mobility. The latter fraction was, consequently, not eluted under the given conditions of the experiment (Fig. 4). The chromatography product, enriched with respect to the first and third fractions, was purified by fraction with ammonium sulfate. The slowest isoenzyme precipitated at a saturation of 0.6–0.67, whereas the fastest isoenzyme at a saturation of 0.75–0.85. The purity of the preparations was checked by acrylamide-gel electrophoresis (Fig. 5).

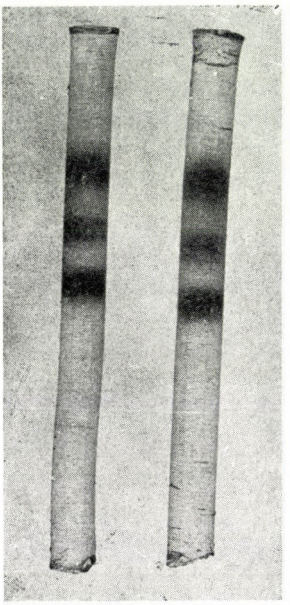

Fig. 3. Electrophoretogram of alkali-treated penicillinase. A, 75 μg penicillinase from constitutive mutant; B, 75 μg penicillinase from constitutive mutant, treated with alkali as described in the text, subsequently adjusted to pH 6.0, run for 3 hours and stained

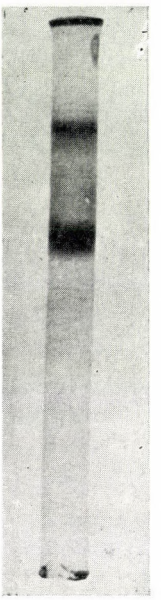

Fig. 4. Electrophoresis of penicillinase from constitutive mutant after chromatography on Celite 545. 70 μg constitutive enzyme chromatographed on Celite 545, separated by electrophoresis for 3 hours and stained

Iodine sensitivity of the penicillinase isoenzymes

A constitutive enzyme preparation was separated by electrophoresis and the isoenzyme fractions cut from the gel were assayed, each, for enzyme activity and iodine sensitivity at pH 6 and pH 9. It has previously been demonstrated that treatment of the enzyme with 10^{-3} N iodine at pH 9 reduced its activity by 30–35%, whereas a similar treatment at pH 6 by only a few per cent. The curves for

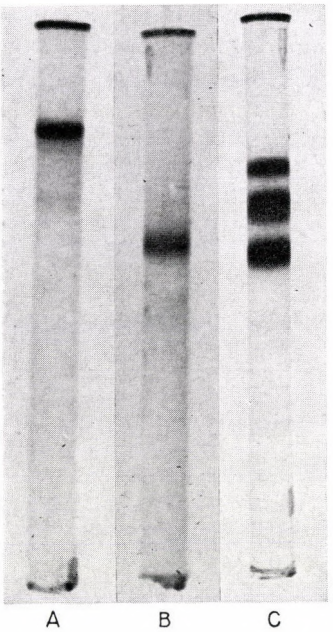

Fig. 5. Electrophoretograms of purified penicillinase isoenzymes. A, Constitutive penicillinase isoenzyme precipitated by saturation with ammonium sulfate (saturation level 0.6—0.67), 30 μg; B, Constitutive penicillinase isoenzyme precipitated by saturation with ammonium sulfate (saturation level 0.75—0.85), 30 μg; C, Untreated mixture of isoenzymes (70 μg). The isoenzymes were separated by electrophoresis for 3 hours and stained

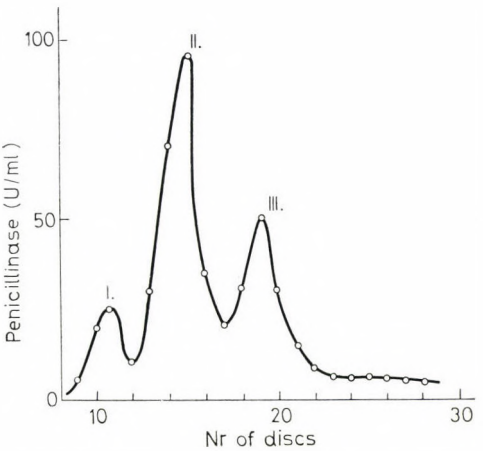

Fig. 6. Activity of constitutive exopenicillinase as measured after electrophoretic separation. The electrophoretogram of the same preparation is shown in Fig. 1

enzyme activity and iodine sensitivity of the preparation are shown in Fig. 6 and in Table 1. The electrophoretogram of the same preparation is shown in Fig. 1. As can be seen from Table 1 the behaviour of the electrophoretically separated isoenzymes toward iodine treatment was similar.

Table 1

Iodine sensitivity of constitutive exopenicillinase as measured after electrophoretic separation

Iodine sensitivities expressed as remaining enzyme activity in per cent of the control samples not treated with iodine

Iodine treatment	at pH 6	at pH 9
	Iodine sensitivities	
Peak I	95	60
Peak II	94	61
Peak III	93	62

Incorporation of iodine by isopenicillinases

Though the preceding experiment served as a fairly convincing proof that the iodine sensitivity of the three isopenicillinases had been similar, additional evidence was sought by examining the behaviour or the three isoenzymes on iodination in the presence of ^{131}I.

Using the method outlined above the penicillinase preparation obtained from the constitutive strain was treated with iodine containing ^{131}I at pH 9 and after subsequent removal of inorganic iodine, electrophoresis was performed. As can be seen from Fig. 7, iodination did not alter the electrophoretic mobility of the isoenzymes. Curves for enzyme activity of the iodinated preparation of Fig. 7 are shown in Fig. 8. The incorporation of radioiodine by penicillinase in peaks I, II, III, were 2.12; 1.91; 2.05 atoms I/mole penicillinase, respectively. Obviously, the three isoenzymes incorporated iodine at almost identical rates. Using a larger amount of enzyme it was demonstrated that with iodination at pH 9 each penicillinase molecule, regardless to which isoenzyme it belonged, incorporated 2 atoms of iodine. In previous studies the formation of 1 molecule di-iodotyrosine had been shown for each molecule of penicillinase.

Reproducing the above experiment with inducible penicillinase similar results were obtained.

Preliminary experiments suggested the presence of one iodinated peptide in the finger-print obtained by trypsin digestion of radioiodine-treated penicillinase.

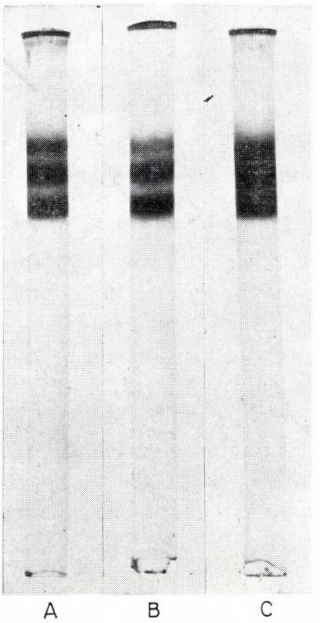

Fig. 7. Electrophoretic mobility of iodinated constitutive enzyme. A, 50 μg constitutive exopenicillinase; B, 50 μg constitutive penicillinase treated at pH 9 with 10^{-3} N iodine containing ^{131}I at 0°C for 1 minute; C, 25 μg constitutive enzyme + 25 μg iodinated constitutive enzyme, separated by electrophoresis for 3 hours and stained

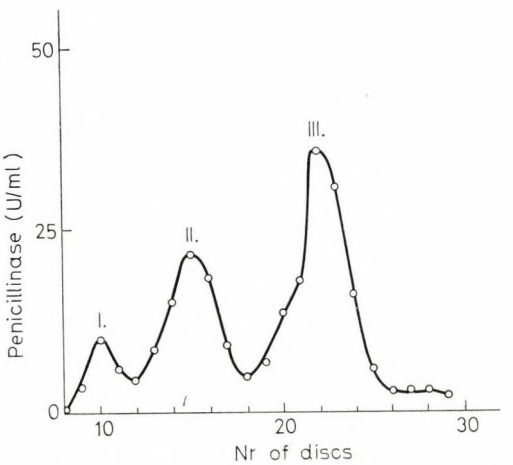

Fig. 8. Enzyme activity of penicillinase treated with ^{131}I. Curve for enzyme activity measured in ån aliquot of the sample shown in Fig. 7B

Discussion

Various conformational changes of penicillinase can be determined by the measurement of the enzyme's iodine sensitivity (Citri and Garber 1958). Inactivation of the enzyme by iodine has previously been shown to take place in two independent steps, one being the "pH-sensitive" reaction which occurred at incubation with 10^{-3} N iodine at pH 9 for 1 minute at 0°C, while the other reaction took place exclusively at pH higher than 9 (Csányi et al., 1970). But these studies did not disclose whether the two reactions were associated with the occurrence of conformational changes or isoenzymes of dissimilar iodine sensitivity, or simply with the inactivation of the penicillinase molecule in two steps.

Experiments with powerful conformation changing agents showed that the two reactions were unrelated with a transition of enzyme conformations with dissimilar iodine sensitivities (Mile et al., 1970). The electrophoretic separability of constitutive exopenicillinase into three isoenzymes was demonstrated by Imsande et al. (1970). This finding was confirmed by reproducing the experiment in this laboratory and in addition it was shown that the penicillinase of the inducible mutant also consisted of 3 isoenzymes. The isoenzymes of the constitutive and inducible strains behaved differently with respect to electrophoretic mobility, as two fractions of each preparation migrated identically, while one of each dissimilarly. Considering that the constitutive strain arose by single-step mutation of the inducible strain, this difference can be regarded very important. Were it due to a diversity of the amino acid sequence, it would be closely related to the regulation of enzyme synthesis. This problem warrants further investigation.

Iodination of the two kinds of enzyme preparations showed that the iodine sensitivities of the individual isoenzymes were similar, regardless whether they originated from the constitutive or the inducible mutant. By means of radioiodine it was demonstrated that on iodination at pH 9, each enzyme molecule incorporated 2 atoms of iodine. Previous spectrophotometric examinations showed that iodine was incorporated in the form of di-iodotyrosine. On the basis of these findings it seems very likely that on iodination at pH 9, viz. in the course of the "pH-sensitive" reaction, each enzyme molecule is iodinated to the same extent and uniformly undergoes a partial loss of activity. Thus the two independent steps of the iodine inactivation reaction of penicillinase represent not so much a "virtual" phenomenon related to the presence of different conformations of the enzyme or isoenzymes, as a common characteristic property of all penicillinase isoenzymes.

The authors are indebted to Professor F. B. Straub for encouragement and valuable advice.

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Examination with Autoradiography of the Intrafibrillar Potassium in Atrophized Muscles

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In order to determine the distribution of intrafibrillar potassium in the unilaterally denervated gastrocnemius muscle of the rat 10 μCi/g of isotonic ^{42}KCl solution was administered intraperitoneally. Three hours later considerable and almost identical ^{42}K activities were measured in the ashes of the gastrocnemius muscles of the two sides. Examination with electron microscopic autoradiography revealed about 25 per cent less grains over the atrophized muscle fibrils than over the normal side muscle fibrils. At the same time the distribution of grains over given sections of the fibril remained unchanged.

This difference in the number of grains is attributed to a loss of muscle potassium as a consequence of atrophy.

Introduction

The fact that the potassium content of the muscle — at least its majority — is localized in the anisotrope band of the muscle fibril is supported by the works of Menten (1908), MacCallum (1911), Winter and Smith (1922), Bureau (1934), Dempsey et al. (1946), Draper and Hodge (1949) and also by the results of several experiments published from this institute (Ernst, 1963; Kállay, Tigyi-Sebes, 1968, 1969; Nesterov, Tigyi-Sebes, 1965; Tigyi-Sebes, 1962). It is also recognized that potassium plays an important role in the muscle activity (Ernst, 1963; Fleckenstein, 1942; Hodgkin, Huxley, 1950; Hodgkin, Horowicz, 1959; Keynes, Lewis, 1950; Tigyi, 1960; etc.). But the problem of the state of potassium in muscle is less unambiguous. In the opinion of several authors (Peters, 1944; Steinbach, 1944; Stone, Shapiro, 1948; Danowski, 1949; Kometiani, 1956 and many others) — and also our institute is of this opinion — the muscle potassium exists, at least in part, in bound state in the anisotrope band of the muscle fibril (see the review of Ernst, 1963). Since the amount of contractile proteins of the muscle decreases after denervation (Straub, 1958), with our experiments performed on the atrophized muscle we hoped to obtain newer data concerning both the state of potassium in the muscle and the possible change of its localization as a consequence of atrophy.

Methods*

In our experiments about 1 cm pieces of the right ischiadic nerve of albino rats was resected behind the femur; the other limb was left intact. After 4 to 16 weeks 10 μCi/g of ^{42}K were given intraperitoneally in the form of isotonic KCl solution. Three hours after the administration of the isotope the animal was killed, and then 2 to 3 pieces of the gastrocnemius muscles of both sides were prepared for electron microscopic examination.

At the same time a certain amount of blood collected during bleeding the animal, and a piece of each of the muscle of both sides were burned to ashes in a Ni crucible after drying.

During histological preparation the fixation was made in vapour of OsO_4 solution for 40 to 60 minutes. The time of dehydration in alcohol-series was decreased to the necessary minimum. After embedding methacrylate the blocks were cut with a Porter–Blum ultramicrotome. The sections mounted on the grids were fixed with a formwar film to a slide and then covered with a film obtained from twice diluted Ilford L-4 emulsion according to the loop method of Caro and van Tubergen (1962) as modified by Caro (1969).

After exposure and developing for 70 to 72 hours photographs of the same magnification and number were taken of the muscles of both sides in a Tesla electron microscope. The electron microscopic autoradiograms were evaluated by counting the grains.

Parallel with the autoradiographic methods the ashes of the blood and muscles were dissolved and the activity of the preparations made in this way was measured.

Results

1. The activity measurements of samples taken from atrophized and normal muscles and of blood ashes revealed ^{42}K administered intraperitoneally to be present in the gastrocnemius muscle in a well measurable quantity 3 hours after the injection. As it is shown by Table 1, building-in is almost of the same degree in the muscles of both sides, and it is almost five times greater than the activity

Table 1

Concentration of ^{42}K in muscle and blood

The data correspond to the values found at the time of the autoradiographic covering.

	μCi/g substance
Atrophized muscle	4.86 ± 0.70
Normal muscle	5.18 ± 0.76
Native blood	1.08 ± 0.16

* Collaborator: A. Tigyi-Sebes.

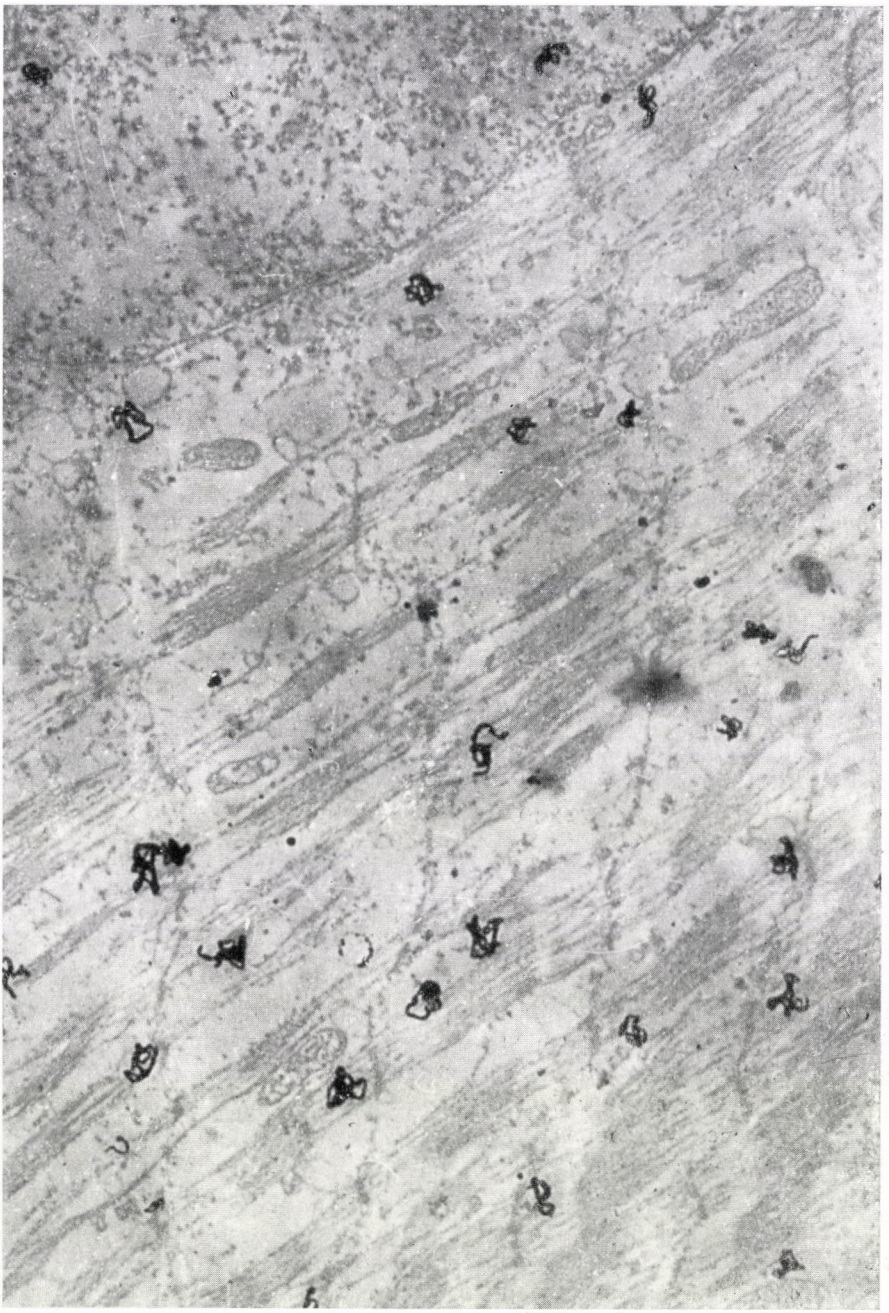

Fig. 1. Electron microscopic autoradiogram of atrophized muscle. Sarcomer length: 2.2 μ. Magnification: 14 800

measured in the blood (the activity was measured the day after the administration, i.e. at the time of the autoradiographic covering). With the complete decay of this activity we hoped to obtain autoradiograms with appropriate numbers of grains, i.e. such ones which can be evaluated.

2. 50 autoradiograms of the same magnification were evaluated on both the atrophized and the normal side muscles (Fig. 1 and Fig. 2). The distribution of grains over the muscle fibril between the sections of the fibril was found to be

Table 2

The numbers of grains and their distributions
(50 evaluated autoradiograms in each case)

	Number of grains				Number of sarcomers
	A-band	I-band	Extrafibr.	Whole	
Atrophized muscle	216 ± 16	145 ± 12	104 ± 10	510 ± 22	2210
Normal muscle	324 ± 18	201 ± 14	113 ± 11	638 ± 25	2195

identical with the distribution previously described for potassium (Kállay, Tigyi-Sebes, 1969), i.e. 2/3 of the grains is situated over the anisotrope band and 1/3 over the isotrope band. This distribution does not change as a consequence of atrophy either. But the number of grains seen in any section of the fibril decreases significantly in muscles with loosened structure due to the atrophy; in these muscles the amount of connective tissue is augmented. This decrease – as it can be seen in Table 2 – amounts to 25 per cent of the grain number found over the normal side muscle.

Discussion

Our experiments performed on atrophized muscle revealed that the ^{42}K activity of the whole muscle does not change significantly as a consequence of the atrophy. This can be attributed to the fact that the circulatory system remained intact during denervation, thus no essential difference exists between the metabolism of the atrophized and of the normal side muscles. However, a significant difference was found in the quantity of potassium situated locally in the muscle fibril. Since disintegration of one structural protein, also observable in the structure, occurs in the muscle after denervation, the decrease of the potassium situated locally in the fibril is also considered to have connection with this protein disintegration. If protein binds potassium in some way, it is evident that with the protein disintegration also potassium releases and, after becoming freely diffusible, it enters the interfibrillar space, from where it can get back into the circulation. In our case, because of the short duration of the experiment, this interfibrillar potassium can be still present in the muscle to a great degree when

Fig. 2. Electron microscopic autoradiogram of normal muscle. Sarcomer length: 2.1 μ. Magnification: 14 800

we measure the activity, but a large part of it can leave with the dehydrating alcohol in the course of dehydration during the histological procedure. Thus, a significant part of autoradiographic grains is offered only by the radioactive potassium still staying in the fibril, the distribution of which between the parts of the fibril agrees with the distribution found in the normal side muscle.

The change in time of the amount of intrafibrillar potassium can give further information, the examination of which is the task of the future.

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Spectral Properties of Protein Solutions Labelled with Fluorescent Dyes

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The dyes fluorescein isothiocyanate and 1-dimethylaminonaphthalene-5-sulfonate are suitable for labelling horse-radish peroxidase, while rhodamine B, rhodamine 6G, rhodulin-orange, eosin, aesculine, trypaflavine and oxytetracycline cannot be used. Spectral data alone are not sufficient to demonstrate the binding of the dyes to the peroxidase, they are useful only together with other methods, e.g. chromatographic analysis.

Introduction

Changes in the secondary, tertiary and quaternary structure of enzymes are investigated among others by measuring changes in the light absorption and fluorescence of enzymes. Three kinds of chromophores and, correspondingly, three kinds of fluorescence are distinguished (Stryer, 1968; Weber, 1952; Chen, 1967): the *intrinsic fluorescence* of chromophores, i.e. the fluorescence originating in the aromatic amino acids of the proteins, *coenzymic fluorescence* due to fluorescence of coenzymes, and *extrinsic fluorescence* due to fluorescent molecules embedded into the protein. The fluorescence depends in each case on the structure of the macromolecule.

The advantages of the extrinsic fluorescence (suggested by Weber, 1952) in investigating the structure of proteins are the generally high yield of fluorescence of bound "dye" and that the fluorescence occurs in the visible region favourable for experimental measurements, whereas the yield of the intrinsic fluorescence is relatively low. Several dyes have been used for labelling proteins. The requirements for dyes to be used in such investigations are as follows: 1. strong, possibly covalent binding to the proteins, 2. intense fluorescence not coinciding with the intrinsic fluorescence of the protein, 3. fluorescence characteristics indicative of the structural changes in the macromolecule and sufficiently sensitive for environmental changes, 4. negligible deformation of the protein molecule by the binding of the dye.

In favourable cases the dye is bound at special (e.g. active) sites and it is the change in fluorescence which gives the most information about the function of the protein. Information is obtained from the fluorescence parameters: emission spectrum, absolute yield, degree of polarization and decay time of fluorescence and absorption spectrum; however, the investigations are mostly restricted to one

of these parameters. In the present paper the changes in the fluorescence characteristics of several dyes forming complexes with enzymes are reported. It was found in many cases that the changes in the fluorescence characteristics are not sufficient to prove the complex formation with the enzyme, therefore such changes are not necessarily due to the enzyme-dye complex.

Materials and methods

Horse-radish peroxidase (HRP, Reanal product) was studied. The activity was determined with iodometric method (Matkovics, Kovács, 1958). The enzyme was purified according to Paul's method (Paul, 1958), lyophilized and stored in a refrigerator. As labelling chromophores rhodamine B (Merck), rhodamine 6G (BDH), rhodulin-orange (Fluka), oxytetracycline (Chinoin), fluorescein-isothiocyanate (in the following FITC, from NBC) and 1-dimethylaminonaphthalene-5-sulfonyl chloride (DNS-Cl, obtained from the Biochemical Institute of the Hungarian Academy of Sciences) were used without further purification.

Absorption spectra were measured with an Optica Milano spectrophotometer (type CF 4 and CF 4 DR). Emission spectra were determined partly with the spectrofluorimeter described by Vize (1959), partly with the UV-spectrofluorimeter built in our laboratory according to the principles given by Ketskeméty and co-workers (1971). A low-pressure mercury lamp was used for excitation. The exciting wavelength was selected by means of a Zeiss quartz prism monochromator (type SPM-2). The fluorescence of the sample was measured perpendicularly to the incident light beam with a Zeiss grating monochromator (type SPM-2). For detection a photomultiplier (EMI 9558 QA) with a galvanometer or a recording compensograph (Zeiss) was used. The emission spectra were corrected for reabsorption; a correction for secondary fluorescence was not necessary (Budó, Ketskeméty, 1957). In order to control the data obtained from spectroscopic measurements, the mixed protein-dye solutions were separated on a Sephadex G 25 column. The dye, with its molecular weight less than that of the protein by two orders of magnitude, diffused together with the enzyme when bound to the peroxidase, whereas the free dye molecules appeared in a separate fraction. The mixed solutions of equimolar dye and protein solutions of 1×10^{-5} M/l concentration were kept at room temperature for 24 hours. Before chromatography, the peroxidase solutions of 1×10^{-4} M/l were incubated with the same volume of 1×10^{-3} M/l dye solutions for 24 hours. Phosphate buffer (pH = 7.0) was used as solvent and eluent. For evaluating the chromatograms the absorption coefficients of the protein at 275 nm and near the absorption maximum of the dye were determined for each fraction or, if the absorption spectra overlapped, the relative intensities near the emission maximum were measured.

Results and discussion

The absorption spectrum $k(\lambda)$ of mixed solutions containing two solutes is equal to the sum of the spectra $k_1(\lambda)$ and $k_2(\lambda)$ of the components when no chemical interaction occurs between the components, whereas in case of a chemical interaction it differs from the spectrum calculated as the sum of the spectra of the components taken up separately. According to Ainsworth and Flanagan

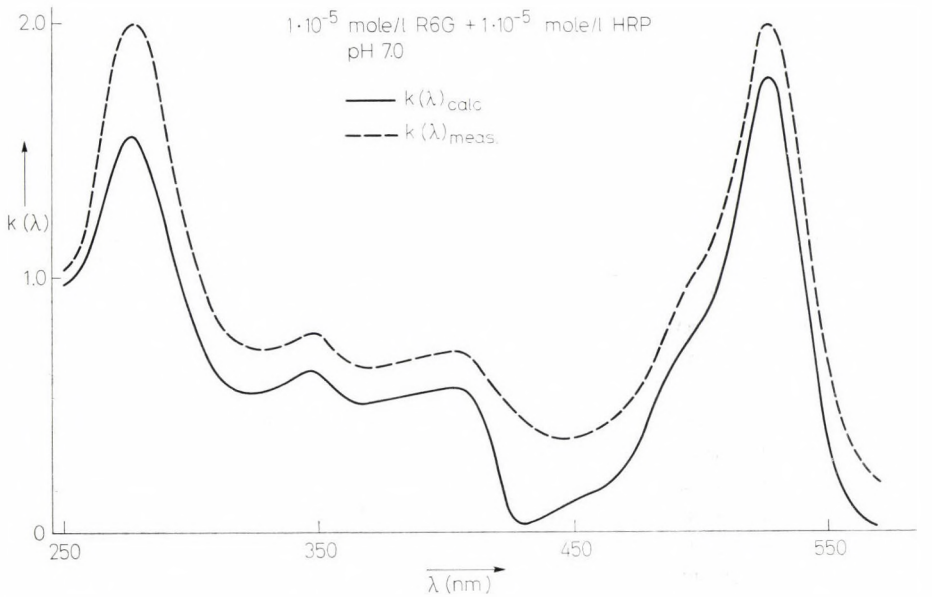

Fig. 1. Absorption spectra of mixed solutions of rhodamine 6G and HRP (——— measured, - - - - - - calculated)

(1969) dyes of rhodamine type are suitable for labelling. In Fig. 1 the absorption spectrum of the equimolar mixed solution of rhodamine 6G and peroxidase is shown by a broken line, the sum of the absorption spectra of the components by a solid line. At the peak characteristic of the peroxidase (280 nm), the difference between the two spectra is greater than at the 520 nm maximum; the sum of the spectra is, however, lower than the absorption spectrum of the mixed solution in the whole range of measurements. A similar behaviour was found for the spectra of mixed solutions containing rhodamine B and rhodulin-orange. The difference was significantly higher than the error of measurement. This ought to point to a chemical binding between the protein and these dyes. This conclusion is, however, not supported by chromatographic and fluorescence measurements. Fig. 2 is a typical elution curve showing that rhodamine 6G is, in fact, not bound to the

peroxidase. The two shoulders and the lower maximum in the chromatogram of the peroxidase may be due to impurities of different molecular weight. The changes in the absorption spectrum found in Fig. 1 cannot result from chemical binding; most probably they are to be accounted for by changes occurring in the environment of the dye molecules during mixing the solutions and, therefore, by changes caused in the absorption by solvent effects. According to our exper-

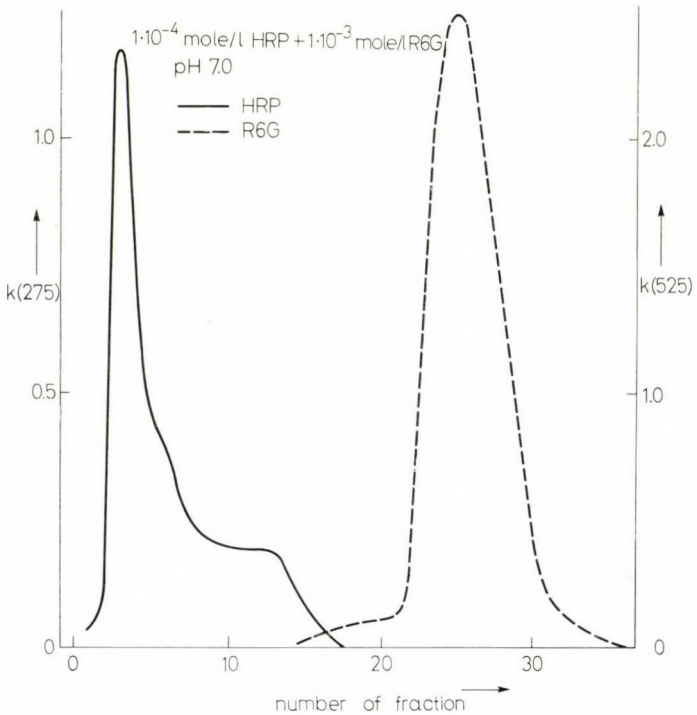

Fig. 2. Elution curve of mixed solutions of rhodamine 6G and peroxidase (——— peroxidase, - - - - - - rhodamine 6G)

iments with dioxane, the absorption coefficients of rhodamine 6G and rhodamine B are increased in apolar solvents, similarly as in the presence of peroxidase. With eosin, aesculine and oxytetracycline as dyes, the differences between the absorption spectra of the mixed solutions and the sum of the spectra of the components do not exceed the range of the experimental errors.

Spectra of different type were found with DNS-Cl and FITC. The spectra of the mixed solutions of protein and of these dyes are markedly lower than the sum of the spectra of the components. In Fig. 3 the broken line shows the absorption spectrum of the mixed FITC and HRP solutions; the sum of the spectra of the components is shown by the solid line. In the case of DNS-Cl and FITC,

significant differences can be seen between the fluorescence spectra of the mixed solutions and the sum of the spectra of single dyes, the intensity of fluorescence in mixed solutions being less than that resulting from the addition of the intensities of the components. Simultaneously the maximum of the fluorescence spectrum of the mixture (broken line in Fig. 4) is shifted towards longer waves by 2.5 nm, when compared with the spectrum of the dye (solid line in Fig. 4). The

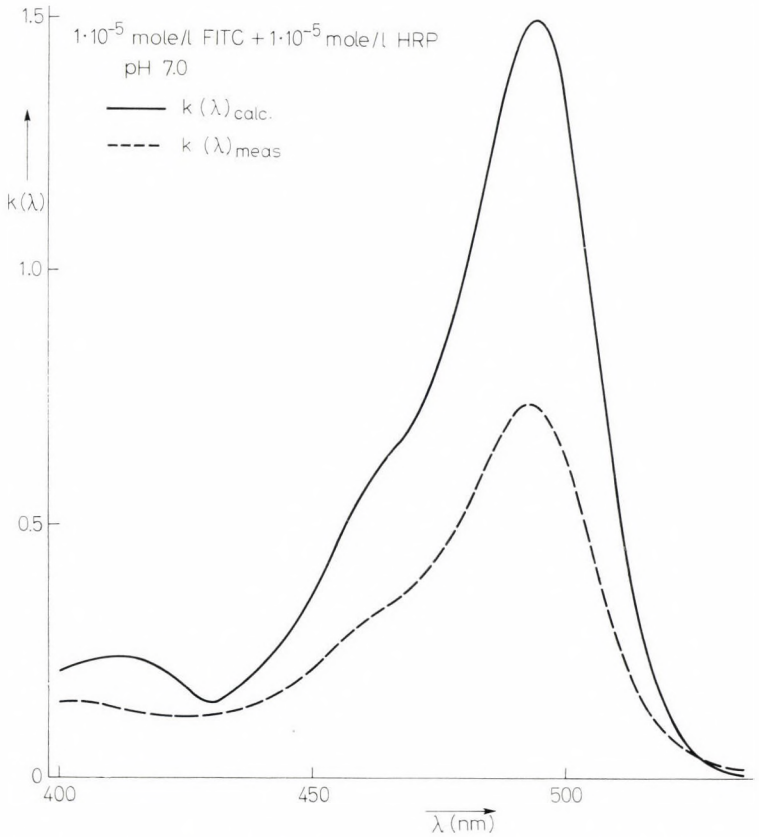

Fig. 3. Absorption spectra of mixed solutions of FITC and HRP
(- - - - - - complex, ——— sum spectrum)

binding of these dyes to the protein is shown also by chromatographic data (Fig. 5). As a result of the excess of the dyes, as well as of the dyes not bound, a separate peak appears about fractions 26 to 28. Thus, in the case of DNS-Cl and FITC both spectroscopic and chromatographic measurements unequivocally point to the formation of a dye–protein complex.

It is concluded from the investigations that fluorescein isothiocyanate and 1-dimethylaminonaphthalene-5-sulfonyl chloride are suitable for labelling the horseradish peroxidase, while dyes of the rhodamine type are not. In addition, the exper-

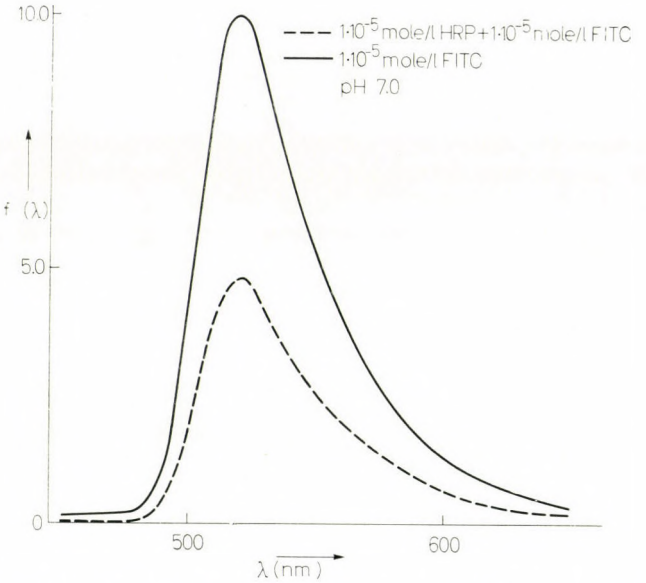

Fig. 4. Emission spectra of FITC and HRP (——— FITC, - - - - - - complex)

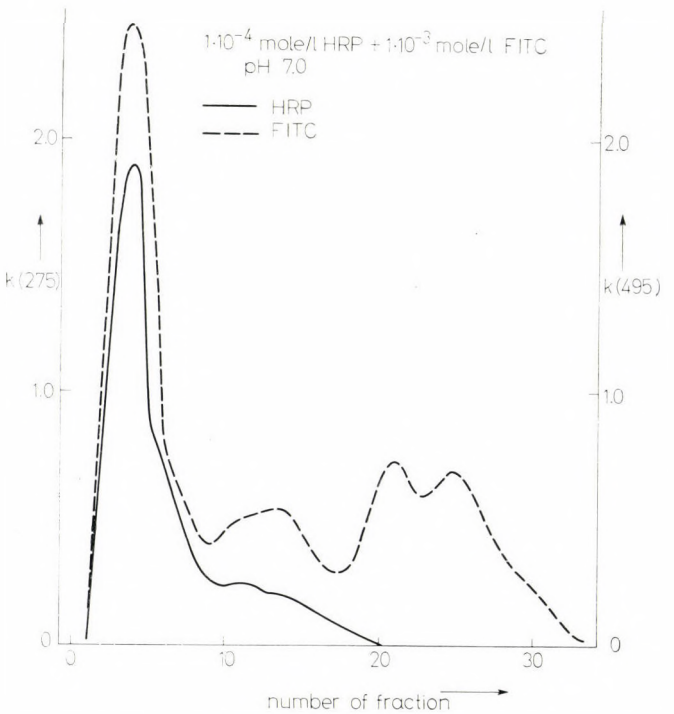

Fig. 5. Elution curve of mixed solutions of FITC and HRP (——— HRP, - - - - - - FITC)

iments proved that the absorption and fluorescence spectra do not provide sufficient proof of a binding between the protein and the dye; therefore, it is not justified to draw conclusions as to the labelling of the protein even from relatively great differences in the spectra, without corroborating the conclusions with other (e.g. chromatographic) methods.

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Potassium Content of Indirectly Stimulated Muscles

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1. It is known that the gastrocnemius muscle of a Läwen–Trendelenburg frog preparation when perfused with normal Ringer's solution loses and does not lose, respectively, from its potassium content upon direct and indirect stimulation. 2. The gastrocnemius stimulated from the plexus did not lose potassium even when the perfusion fluid contained narcotics (urethane, chloralose, barbital, paraldehyde, chloralhydrate). 3. One of the frog preparations was perfused with Ringer's solution flowing out from another stimulated preparation, in order to examine the potential role of the fatigue substance possibly going together with the stimulation. The indirectly stimulated gastrocnemius of the former one did not lose potassium either. 4. The gastrocnemius of the frog anaesthetized by injecting narcotics into the lymphatic sack did lose potassium upon the effect of direct stimulation but not in the case of an indirect stimulation. Conclusion: *the excitation of the muscle does not go together with* a *decrease of potassium content.*

Introduction

After demonstrating experimentally first in the literature (Ernst, Scheffer, 1928; Ernst, Csucs, 1929) that the frog gastrocnemius perfused with Ringer's solution loses K and takes up Na when stimulated directly, but it does not when stimulated indirectly, Ernst et al. stated that *permeability does not increase upon the effect of indirect stimulus, i.e. during physiological excitation* (see Ernst, 1963). These experimental results have been soon corroborated (Mond, Netter, 1930; Dubuisson, 1934; Fenn, Cobb, 1934, 1936; Fenn, 1936)* but, according to later experiments, a K-loss appears also in a muscle stimulated indirectly. The indirectly stimulated muscle of anaesthetized rat (Fenn, Cobb, 1936), cat (Fenn et al., 1938) and dog (Wood et al., 1939—1940) releases K into the circulating blood during a series of indirect stimulation till fatigue; furthermore the gastrocnemius of the anaesthetized frog also loses K (Fenn, 1937) under similar circumstances.

As the main question is the supposed increase of permeability during *physiological excitation*, the latter experimental results cannot be evaluated because

* Beside these facts it should be also mentioned that one year later Fenn quotes only himself (Fenn, 1937a) to the fact: "that in electrically stimulated muscles of . . . frogs there is a loss of potassium in exchange of sodium . . .". The statement (Wilde et al., 1955) that: "Fenn and Cobb *detected* losses of potassium from muscle after . . . stimulation" arises logically from this.

it was demonstrated already by Mosso (1890) that a muscle perfused with blood coming from a tired muscle shows the signs of fatigue. However, in each of the enumerated series of experiments the "substance of fatigue" could be accumulated in the circulating blood.

On the basis of the above data the question was examined whether the indirectly stimulated gastrocnemius of anaesthetized frog loses potassium a) if the change of solution is assured by perfusion and b) if – in the case of closed blood circulation – the excessive fatigue of the muscle is avoided. Under such experimental conditions the indirectly stimulated frog gastrocnemius did not lose potassium, as opposed to the above data which served as a basis for the assumption that the physiological excitation of the muscle goes together with an increase of permeability.

Methods*

Several methods were applied in order to solve the biological problem raised here.

1. A series of experiments was performed with 2 frogs each. Both sides of Läwen–Trendelenburg frog preparations were perfused with normal Ringer's solution. The left ischiadic plexus of one of the frogs was stimulated with induced current by means of a metronome for 0.3 sec at every 2 sec. Alternating current of 2 V and 50 Hz was given to the primary coil and the stimulating voltage was taken off at a 30–38 cm position of the secondary coil (tetanus). The m. gastrocnemius of the other frog was directly stimulated with alternating current of 2 to 20 V. The stimulation lasted 20 to 35 minutes in each experiment. The direct or indirect stimulation was always performed until fatigue.

2. In another series of experiments the frog preparations were perfused with normal Ringer's solution containing narcotics. The quantity of narcotics: urethane 2 to 4 g, chloralose 0.03 to 0.06 g, barbital-Na 0.25 to 0.50 g, paraldehyde 1 to 2 g and chloralhydrate 0.15–0.60 g per liter. The left ischiadic plexus of the frogs was stimulated in the way described in paragraph 1.

3. In the third series of experiments we wished to examine whether the potassium content of the indirectly stimulated muscle is influenced by perfusion with a solution that flowed out from a stimulated muscle preparation. For this purpose (Ernst, 1932) the frog preparations were perfused with normal Ringer's solution and with solution containing narcotics, resp., in a two-storied arrangement. (The quantity of the narcotics agrees with that described in paragraph 2.) The perfusion-solution was led into the dorsal aorta of the upper frog, the solution perfused through the frog was led into the dorsal aorta of the lower frog through the abdominal vein. So the lower frog always got the solution which flowed through the upper frog. The ischiadic plexus of both frogs was stimulated in series.

4. In the fourth series of experiments 1 ml of narcotics was injected into the lymphatic sack of the frog (urethane 4 g, barbital-Na 0.7 g, paraldehyde 2 g

* Collaborator: K. Gábor.

and chloralhydrate 0.6 g each per kg). When the frog got into narcotic state (it had only cornea reflex) the right thigh was strongly ligated in the middle and the left gastrocnemius muscle was stimulated either directly (through autoformer, raising the stimulating voltage gradually from 2 V to 40 V) or indirectly (as above).

The quantity of the solution flowing through the preparations was measured in each case. The K – Na – Ca contents of the muscles were determined with a Zeiss flame-photometer after drying and burning the muscles (at 450 °C in an electrical stove). The composition of the normal Ringer's solution used in our experiment was: 6.6 g NaCl, 0.2 g KCl, 0.2 g $CaCl_2$ sicc, 0.2 g $NaHCO_3$ in 1000 ml of bidistilled water.

Results

1. First it was examined how the potassium content of the muscle changes if the frog preparation is perfused with normal Ringer's solution and the m. gastrocnemius is stimulated directly and indirectly, respectively. Table 1 shows that no essential difference can be found between the potassium content of the indirectly stimulated and control muscles, while in the case of direct stimulation a difference of about 10 per cent is observed. (The average wet weight of the indirectly stimulated muscles is 1.15 g, that of the controls is 1.39 g; in the case of direct stimulation 1.38 g and 1.32 g.)

2. In the second part of our experiments it was examined how the potassium content of the muscle is influenced during indirect stimulation by the narcotics

Table 1

Perfusion of Läwen–Trendelenburg frog preparation with normal Ringer's solution
Indirect stimulation on the P. ischiadicus and direct stimulation on the M. gastrocnemius

No.	Potassium content of M. gastrocnemius in mg			
	indirect stim.	control	direct stim.	control
1	4.76	4.76	4.00	4.28
2	4.68	4.68	4.12	4.52
3	3.48	3.64	4.08	4.40
4	4.40	4.48	3.80	4.12
5	5.76	5.84	3.56	3.80
6	4.12	4.20	4.12	4.32
7	4.64	4.72	3.84	4.28
8	4.72	4.80	2.96	3.48
9	4.08	4.16	2.76	3.48
10	4.60	4.68	3.56	4.08
11	3.60	3.60	4.44	5.12
12	4.72	4.76	4.40	4.80
	K = 4.46	K = 4.52	K = 3.80	K = 4.22

Table 2

Perfusion of Läwen—Trendelenburg frog preparation with normal Ringer's solution containing narcotics
Indirect stimulation on the P. ischiadicus

No.	Potassium content of M. gastrocnemius in mg									
	urethane		chloralose		barbital-Na		paraldehyde		chloralhydrate	
	stim.	contr.	stim.	contr.	stim.	contr.	stim.	contr.	stim.	contr.
1	5.52	5.52	4.68	4.80	3.88	3.88	4.76	4.68	5.60	5.50
2	5.04	5.16	5.36	5.56	3.88	4.16	5.00	5.08	6.65	6.60
3	5.00	5.00	4.68	4.72	5.16	5.16	5.40	5.40	5.85	5.85
4	5.60	5.68	3.42	4.32	4.76	4.80	4.28	4.32	6.50	6.65
5	4.40	4.44	4.40	4.40	4.60	4.68	—	—	7.00	7.00
6	4.00	4.08	4.72	4.80	4.96	4.96	—	—	5.20	5.30
7	3.40	3.28	4.80	4.80	4.76	4.88	—	—	5.40	5.50
8	3.72	3.72	5.48	5.60	4.68	4.68	—	—	5.55	5.55
9	3.64	3.64	4.28	4.40	4.40	4.44	—	—	—	—
10	3.96	3.96	5.20	5.20	3.48	3.36	—	—	—	—
11	3.64	3.64	4.40	4.40	5.28	5.28	—	—	—	—
12	4.64	4.72	4.36	4.36	5.40	5.48	—	—	—	—
13	4.16	4.16	—	—	—	—	—	—	—	—
14	4.40	4.40	—	—	—	—	—	—	—	—
15	4.20	4.20	—	—	—	—	—	—	—	—
16	2.80	2.80	—	—	—	—	—	—	—	—
	K = 4.26	K = 4.27	K = 4.72	K = 4.78	K = 4.60	K = 4.64	K = 4.86	K = 4.87	K = 5.97	K = 5.99

given to the perfusion solution. (The average wet weight of the muscles: stimulated: 1.53 g, control: 1.45 g.) These results are shown in Table 2. The average potassium content of the stimulated muscles is 4.88 mg, that of the control is 4.91 mg. According to this no significant difference can be demonstrated between the potassium content of the indirectly stimulated and not stimulated muscles of anaesthetized frogs.

3. The results of our perfusion experiments in two-storied arrangement are shown in Table 3; on the basis of these data no difference was found between the potassium content of the indirectly stimulated and control muscles: the average potassium content of the stimulated muscles was 5.24 mg, and that of the control muscles 5.23 mg. (Average wet weight of muscle: stimulated 1.77 g, control 1.65 g.)

Table 3

Gradual perfusion of Läwen–Trendelenburg frog preparation with normal Ringer's solution and normal Ringer's solution containing narcotics

Indirect stimulation through the P. ischiadicus

No.	Potassium content of M. gastrocnemius in mg							
	normal Ringer		urethane		chloralose		barbital-Na	
	stim.	contr.	stim.	contr.	stim.	contr.	stim.	contr.
	Upper frog							
1	4.88	4.80	4.60	4.60	6.40	6.40	5.05	5.00
2	5.36	5.44	5.60	5.60	4.40	4.25	4.60	4.50
3		—	6.25	6.25	—	—	—	—
4	—	—	7.40	7.40	—	—	—	—
	K = 5.12	K = 5.12	K = 5.96	K = 5.96	K = 5.40	K = 5.33	K = 4.82	K = 4.75
	Lower frog							
1	5.04	5.04	4.48	4.44	4.65	4.75	5.65	5.65
2	4.88	4.88	5.08	5.08	4.80	4.70	5.95	6.05
3	—	—	5.90	5.90	—	—	—	—
4	—	—	5.20	5.20	—	—	—	—
	K = 4.96	K = 4.96	K = 5.16	K = 5.15	K = 4.72	K = 4.72	K = 5.80	K = 5.85

4. At last it was examined how the potassium content of the muscle changes in the case of direct and indirect stimulation if the frog is anaesthetized by narcotics injected into the lymphatic sack. The potassium content of the stimulated muscles is 3.28 mg on the average, that of the controls is 3.60 mg. The average wet weight of the stimulated and control muscles is 1.22 g and 1.13 g, respectively. Accord-

ing to these data (Table 4a) some 10 per cent difference can be found in the potassium content of directly stimulated and control muscles. With indirect stimulation (Table 4b) there is no significant difference between the potassium content of stimulated and control muscles: the potassium content of stimulated muscles is 2.84 mg on the average, that of the control muscles is 2.88 mg. (Average wet weight of muscle: stimulated 0.97 g, control 0.94 g.)

Table 4a

Narcotization of Rana esculenta with narcotics injected into the lymphatic sack
Direct stimulation on M. gastrocnemius

No.	Potassium content of M. gastrocnemius in mg							
	urethane		barbital-Na		chloralhydrate		paraldehyde	
	stim.	contr.	stim.	contr.	stim.	contr.	stim.	contr.
1	2.69	3.23	3.33	3.74	3.24	3.30	3.68	4.06
2	3.30	3.42	2.94	3.42	2.91	3.18	3.20	3.49
3	2.50	2.91	3.04	3.74	3.88	4.16	3.87	4.00
4	3.65	4.10	3.10	3.49	4.32	4.52	2.85	3.00
	K = 3.05	K = 3.41	K = 3.10	K = 3.58	K = 3.59	K = 3.79	K = 3.40	K = 3.64

Table 4b

Narcotization of Rana esculenta with narcotics injected into the lymphatic sack
Indirect stimulation through the N. ischiadicus

No.	Potassium content of M. gastrocnemius in mg							
	urethane		barbital-Na		chloralhydrate		paraldehyde	
	stim.	contr.	stim.	contr.	stim.	contr.	stim.	contr.
1	2.84	2.88	2.34	2.28	4.80	4.80	2.11	2.09
2	2.76	2.76	2.43	2.43	4.38	4.44	2.09	2.11
3	3.40	3.28	2.61	2.61	2.48	2.56	2.70	2.70
4	2.88	2.96	2.94	3.00	2.23	2.27	3.06	3.08
	K = 2.97	K = 2.97	K = 2.58	K = 2.58	K = 3.48	K = 3.51	K = 2.49	K = 2.49

Discussion

The first paper dealing with this question already formulated the results (Ernst, Csucs, 1929) as follows (p. 666): "*as the electrolyte loss is missing in the case of indirect stimulation*, we must suppose that the *increase of permeability did not occur*." But according to the present standpoint of the majority the increase of permeability is the basic phenomenon of *physiologic excitation*, which would be proved by the result of the works about the potassium loss of the indirectly stimulated muscle cited above. However, the muscle was not examined in *physiologic* state in either of these experiments, but was damaged by anaesthesia, excessive fatigue, etc. Damaging factors are, in fact, mentioned by several authors in order to explain the K-loss found in the case of indirect stimulation (e.g. Creese et al. 1958; anoxia). But this kind of damage cannot yet occur at the beginning of the action in the muscle that became excited from the direction of the nerve, thus its permeability did not increase either and still produced an action current of normal value.

On the other hand the electrolyte loss occurring after direct stimulation of the muscle can partly be attributed to the damaging effect of direct current* (Ernst, 1963). This is corroborated by experiments (Hodgkin, Horowitz, 1959) in which the electrolyte change increased during direct stimulation of an isolated muscle fibre, though in the author's opinion the change "produced by stimulation can safely be ascribed to propagated action potentials and not to local effects . . .". On the other hand, hardly anybody wants to question that the electric current flowed through the stimulated muscle fibre between the two electrodes.

Up to now we did not find data in the literature which would justify the assumption that the physiologic excitation of the muscle would be accompanied by an increase of permeability.

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* See similar arguments concerning the nerve (Ernst, 1963).

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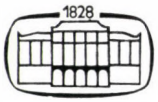

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Heat Inactivation of D-glyceraldehyde-3-phosphate Dehydrogenase Apoenzyme

MÁRIA VAS, L. BOROSS

Enzymology Department, Institute of Biochemistry, Hungarian Academy of Sciences Budapest

Dedicated to Professor A. E. Braunstein in commemoration of his 70th birthday

(Received January 24, 1972)

The rate constant of heat inactivation of pig muscle D-glyceraldehyde-3-phosphate dehydrogenase apoenzyme depends on the concentration of the enzyme. It is assumed that the marked decrease in stability below 0.1 mg/ml protein concentration reflects the dimer–monomer dissociation of the tetrameric enzyme. The dissociation constants estimated from the concentration dependence of the rate constants of inactivation at 30, 40 and 45 °C are 3×10^{-9}, 8×10^{-9} and 2×10^{-8} M, respectively, in 0.05 ionic strength tris/HCl buffer pH 8.5, in the presence of 2-mercaptoethanol. For the dissociation process between dimers and monomers $\Delta H = 24$ kcal/mole and $\Delta S = 40$ cal/degree × mole values were obtained.

The activation energy of heat inactivation of the monomeric enzyme form is about 30 kcal/mole.

Hybrid formation between inactive carboxymethylated and active subunits has been demonstrated.

Introduction

It was reported recently (Lakatos et al., 1971; Hoagland, Teller, 1969; Spotorno, Hollaway, 1970) that tetrameric D-glyceraldehyde-3-phosphate dehydrogenase (GAPD) dissociates into subunits on dilution. Dissociation of mammalian GAPD into monomers (Lakatos et al., 1971) and dimers (Lakatos et al., 1971; Hoagland, Teller, 1969) has been shown by sedimentation equilibrium technique. Dissociation into dimers has also been confirmed by the hybridization of rabbit muscle and yeast GAPD (Spotorno, Hollaway, 1970).

It has been shown that the heat stability of GAPD changes with enzyme concentration (Vas, Boross, 1970). From the above data we supposed that this phenomenon reflects the dissociation of the enzyme molecule, i.e. the stability of the enzyme depends on the degree of association.

The aim of this work was to study in detail the concentration dependence of heat stability of GAPD apoenzyme in order to characterize the dissociation process of the tetrameric enzyme. Beside the sedimentation equilibrium technique heat inactivation is another independent method for detecting the dissociation of an oligomeric enzyme. By this test the effect of temperature on dissociation can be studied at a few μg/ml enzyme concentration.

Materials and methods

Four times recrystallized pig muscle GAPD (Elődi, Szörényi, 1956) was used. The enzyme was gel-filtered before each experiment on a Sephadex G – 100 column equilibrated with 0.05 ionic strength tris/HCl buffer, pH 8.5, to remove ammonium sulfate.

The experiments were carried out with the apoenzyme, i.e. the tightly bound coenzyme was removed by charcoal treatment (Velick, 1953), which resulted in an increase of E_{280}/E_{260} ratio from 1.05 to 1.85 – 1.95.

All solutions contained 5×10^{-3} M 2-mercaptoethanol to prevent oxidation of the thiol groups of the enzyme. The concentration of 2-mercaptoethanol was checked during heat treatment.

Specific activity of the enzyme at 20 °C varied between 12 000 and 15 000 moles NADH $\times$ min^{-1} $\times$ mole^{-1}. The molecular weight of the tetrame r was taken as 145 000 (Elődi, 1958).

Heat inactivation was monitored by incubating enzyme solutions of various concentrations at different temperatures and by assaying activity at appropriate time intervals.

Enzyme activity was assayed with 1 μg/ml enzyme at the temperature and pH at which the incubation was performed. In the assay mixture the enzyme was saturated with substrates. Activity was calculated from the first 30 sec of enzyme reaction. Zero time values were determined at each temperature.

Measurements were carried out in a Unicam SP 700 spectrophotometer equipped with thermostated cell compartment.

The change of pH of buffer solutions with temperature was taken into account.

Glyceraldehyde-3-phosphate was prepared from fructose-1,6-diphosphate (Reanal) according to Szewczuk et al. (1961). NAD was the product of Reanal. The other chemicals were commercial preparations of reagent grade.

Results and discussion

1. *Time course of heat inactivation at different temperatures.* Heat inactivation of GAPD was determined at 30, 40 and 45 °C. Inactivation did not follow simple first order kinetics, in the semilogarithmic plot downward concave curves were obtained (Fig. 1). Furthermore, the initial rate constant of inactivation depends on protein concentration: the more diluted the solution, the higher is the rate constant.

We assume that the time course of inactivation and the concentration dependence of the initial rate constant of inactivation are due to a dissociating system in which the dissociated forms are more labile than the tetramer. This assumption is also supported by the fact that bovine serum albumin in 1 mg/ml concentration has no effect on the kinetics.

The kinetic analysis of the time course of inactivation will be presented in section 4.

2. *Estimation of dissociation constants.* From the initial parts of the progress curves of heat inactivation one may estimate the initial dissociation equilibrium of the enzyme. The first order rate constants calculated from the initial, practically linear parts of the semilogarithmic plots were plotted against enzyme concentration (Fig. 2). The data show that the rate constant of inactivation markedly increases below 0.1 mg/ml enzyme concentration.

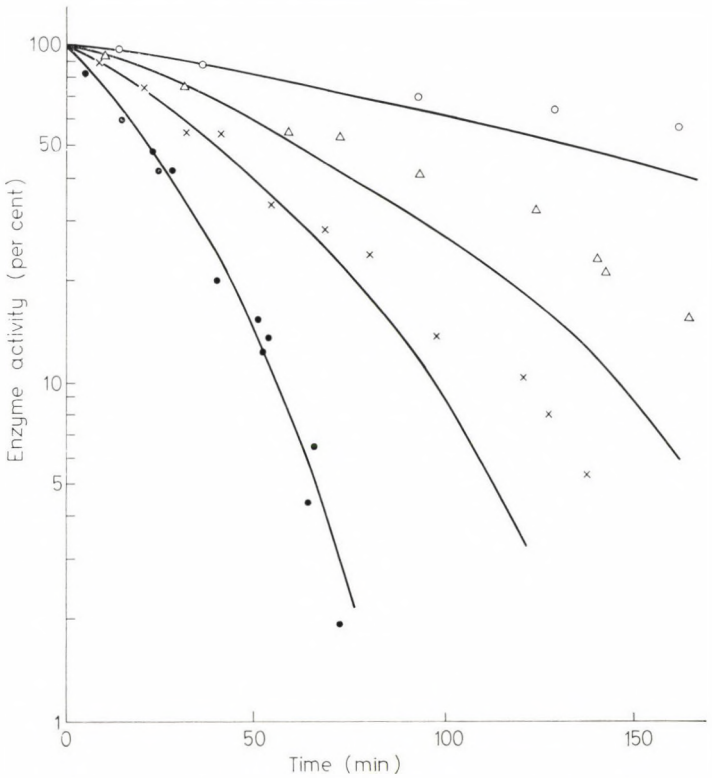

Fig. 1. Time course of heat inactivation of GAPD apoenzyme. Experimental conditions: 0.05 ionic strength tris/HCl buffer, pH 8.5, containing 5×10^{-3} M 2-mercaptoethanol at 40 °C. 1.2 μg/ml (• — •), 4.8 μg/ml (× — ×), 12 μg/ml (△ — △) and 60 μg/ml (○ — ○) GAPD concentrations. The solid lines are theoretical curves calculated from equation (3), with constants listed in Table 1

The data in literature (Lakatos et al., 1971; Hoagland, Teller, 1969) suggest that dissociation of GAPD tetramer occurs in two steps: the tetramer dissociates into dimers and the dimer into monomers. From these data it seems very probable that at very low enzyme concentrations (a few μg per ml) we deal mainly with the dimer–monomer dissociation process. Therefore we neglected the amount of the

tetrameric form below 50 μg/ml concentration. The method described in the Appendix was used for estimating both the dissociation constant and the rate constants of inactivation of dimeric and monomeric forms. The experimental data presented in Fig. 2 fit the theoretical curves satisfactorily only if the rate constant of inactivation of the dimeric form is lower than that of the monomeric form with more than one order of magnitude. Therefore in the calculations inactivation of the dimeric form need not be considered. The dissociation constants for dimer–

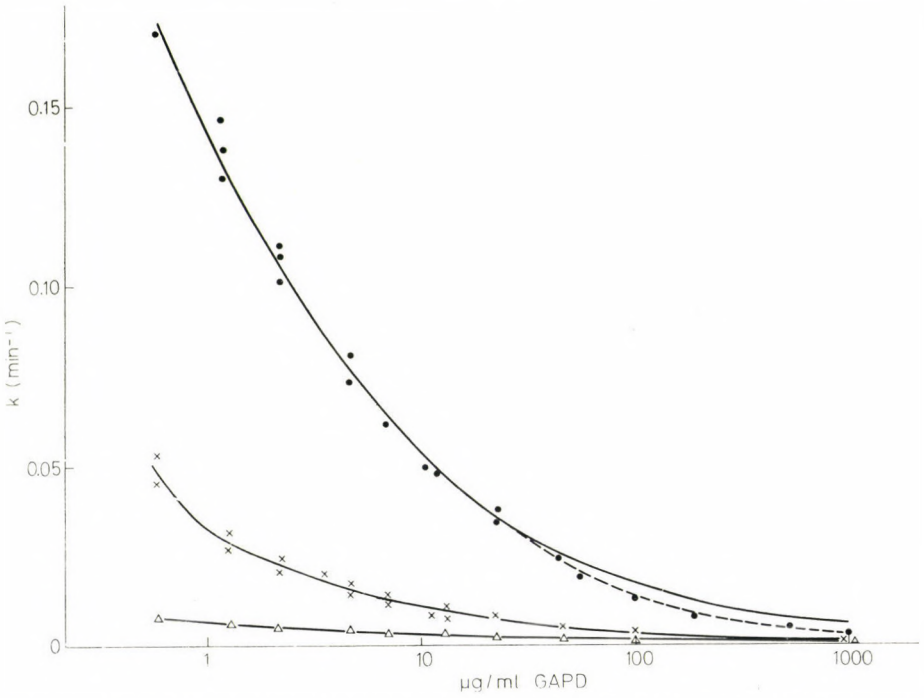

Fig. 2. Dependence of the rate constant of heat inactivation on enzyme concentration. The ordinate represents the first order rate constants calculated from the initial phase of inactivation at 30 °C (△—△), 40 °C (×—×) and 45 °C (•—•). The experimental conditions are the same as in legends to Fig. 1. The solid lines are theoretical curves which correspond to a dimer–monomer dissociation equilibrium, calculated with the constants given in Table 1. The broken line is a theoretical curve corresponding to a tetramer–dimer–monomer system at 45 °C, calculated with the dissociation constants for dimer–monomer equilibrium given in Table 1 and 10^{-6} M for the tetramer–dimer dissociation

monomer equilibria and the rate constants of inactivation of the monomeric form obtained by this method are seen in Table 1, and the continuous lines in Fig. 2 represent dissociation curves calculated with these values.

At enzyme concentrations higher than 50—100 μg/ml the measured rate constants somewhat deviate from those predicted by the dimer–monomer equilibrium. This is indeed to be expected, as at higher enzyme concentrations the

tetramer–dimer equilibrium is also called into play. Assuming a value of 10^{-6} M for the tetramer–dimer dissociation constant and that the rate of inactivation of the tetramer is also negligible, we calculated the theoretical dissociation curve at 45 °C for the tetramer–dimer–monomer system. Derived from the calculated monomer concentration, the broken line in Fig. 2 corresponds to the theoretical dependence of the rate constants on enzyme concentration. This curve agrees well with the experimental points in the whole concentration range investigated. This

Table 1

Thermodynamic parameters of dimer–monomer dissociation of GAPD apoenzyme

pH = 8.5 tris/HCl buffer, ionic strength = 0.05 in the presence of 5×10^{-3} M 2-mercaptoethanol

	30 °C	40 °C	45 °C
Rate constant of inactivation of monomer (min^{-1})	0.027	0.095	0.33
Dissociation constant (M)	3×10^{-9}	8×10^{-9}	2×10^{-8}
ΔG (kcal/mole)	11.8	11.6	11.2

ΔH = 24 kcal/mole
ΔS = 40 cal/degree mole

also supports the idea that the observed concentration dependence of heat inactivation is primarily due to the dimer–monomer dissociation process.

3. *Thermodynamic parameters of dimer–monomer dissociation and the stability of the monomer.* The dissociation constant of the dimer–monomer equilibrium depends on temperature. The van't Hoff plot of dissociation constants gives a straight line (Fig. 3). From the slope of this line 24 kcal/mole was obtained for the enthalpy change of dissociation. The positive value indicates that under the experimental conditions used the monomers are held together in the dimers mainly by electrostatic forces (Scheraga, 1961).

From the temperature dependence of the dissociation constants the Gibbs free enthalpy (ΔG) and entropy (ΔS) changes of dissociation were also calculated (Table 1).

In order to characterize the structural stability of monomer we calculated the activation energy needed for inactivation of the monomeric form from the rate constants listed in Table 1. The temperature dependence of the rate constants gives a linear Arrhenius plot (Fig. 4) and the activation energy for inactivation of the monomeric form is about 30 kcal/mole. This value is practically the same if 0.1 M glycine buffer containing 0.1 M NaCl is used instead of tris/HCl buffer.

It should be noted that these considerations do not imply that the enzyme is active in the monomeric form, because we do not know which form of the enzyme (monomer, dimer or tetramer) is predominant under the conditions of activity

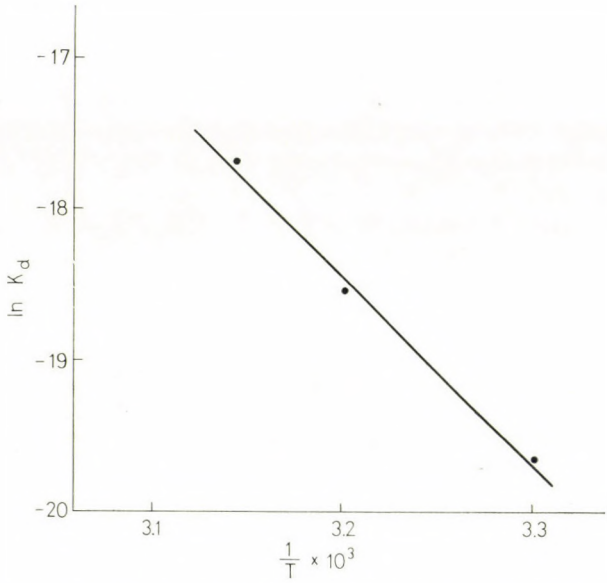

Fig. 3. Temperature dependence of equilibrium constants of dimer–monomer dissociation (Van't Hoff plot). The equilibrium constants were estimated as described in the Appendix and are given in Table 1

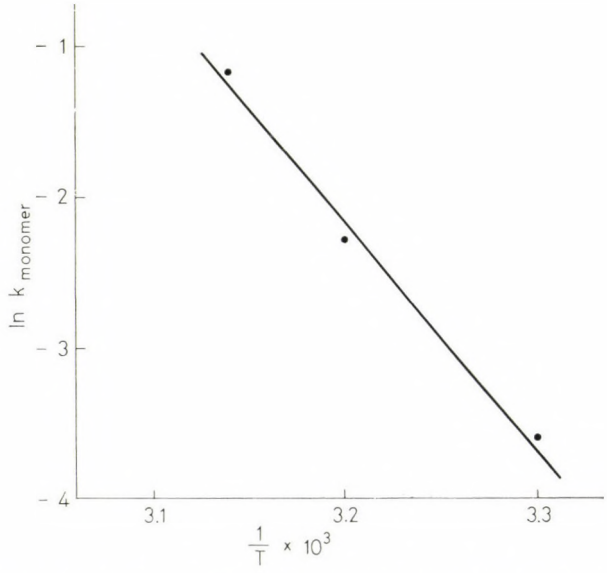

Fig. 4. Temperature dependence of the rate constants of heat inactivation of GAPD monomer (Arrhenius plot). The rate constants were estimated as described in the Appendix and are given in Table 1

assay, i.e. how the presence of substrates influences the association – dissociation equilibrium.

4. *Kinetic analysis of the time course of heat inactivation.* The enhanced lability of the monomeric form in the above dissociating system explains not only the concentration dependence of inactivation but also the deviation of the time course of inactivation from first order kinetics. If we assume that (i) association–dissociation is much faster than inactivation (rapid equilibrium), (ii) the rate of inactivation of dimers is negligible as compared to that of monomers and (iii) the inactivated forms cannot associate with active forms, then inactivation will become faster as the concentration of active enzyme decreases. In other words the extent of dissociation will increase during the course of inactivation process.

Using the dissociation constants estimated from the experimental data we calculated the theoretical time course of inactivation in the following way:

The rate of inactivation can be written:

$$\frac{\mathrm{d}[E]}{\mathrm{d}t} = \frac{\mathrm{d}[m]}{\mathrm{d}t} = -k_{\text{monomer}}\,[m] \tag{1}$$

where $[E]$ = molar concentration of the active enzyme at time t calculated for the subunit molecular weight 36 125,

$[m]$ = molar concentration of monomer in the active enzyme at time t,

t = time,

k_{monomer} = first order rate constant of inactivation of the monomeric form.

From the equation of dissociation equilibrium it follows that

$$\frac{\mathrm{d}[E]}{\mathrm{d}t} = -k_{\text{monomer}}\,\frac{-K_d \pm \sqrt{K_d^2 + 8K_d\,[E]}}{4} \tag{2}$$

where K_d = dissociation constant of dimer–monomer equilibrium. By integrating this equation and taking only the positive square root into account we obtain

$$\frac{1}{K_d}\left(\sqrt{K_d^2 + 8K_d\,[E]} - \sqrt{K_d^2 + 8K_d\,[E]_0}\right) + \ln\frac{\sqrt{K_d^2 + 8K_d\,[E]} - K_d}{\sqrt{K_d^2 + 8K_d\,[E]_0} - K_d} = -k_{\text{monomer}}\,t \tag{3}$$

where $[E]_0$ is the value of $[E]$ at $t = 0$.

In dilute solution the time course of inactivation agrees well with the calculated curve (cf. Fig. 1). However, the more concentrated the solution, the greater the deviation from the theoretical curve. This indicates that inactivation is a more complex process and the partially denatured inactivated monomers may also combine with active monomers. The formation of hybrids of active and inactive subunits may influence the rate of further inactivation, as such hybrids may have different dissociation constants, and their rate of inactivation probably also differs from that of native enzyme, etc.

We should like to mention that in the absence of 2-mercaptoethanol inactivation follows first order kinetics up to about 95% inactivation. The rate constants are higher and also depend on enzyme concentration. The dissociation constants for dimer–monomer equilibria as calculated from this concentration dependence are also higher (Vas, Boross, 1971) than in the presence of 2-mercaptoethanol. This finding indicates that oxidation of SH-groups is involved in the inactivation in the absence of reducing agent.

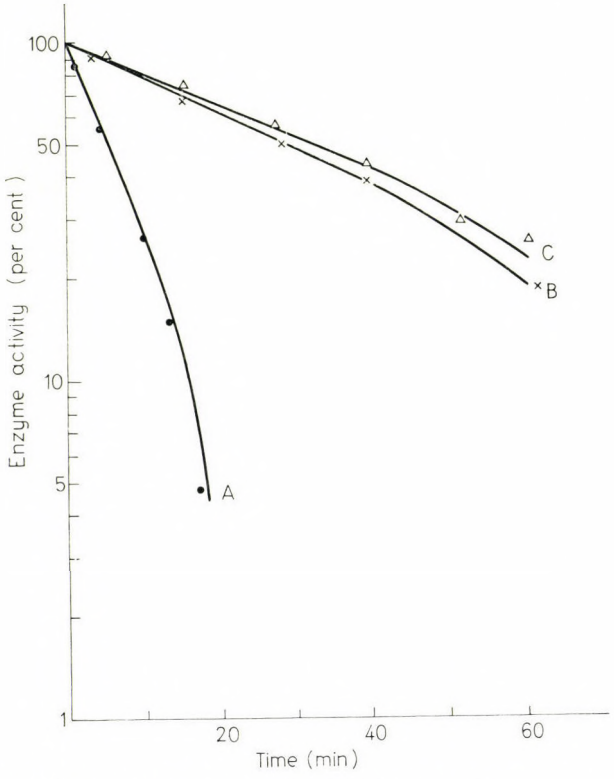

Fig. 5. Time course of heat inactivation of GAPD in the presence of carboxymethylated enzyme. Curve A, inactivation of GAPD at 1.2 μg/ml; Curve B, inactivation of GAPD at 1.2 μg/ml, in the presence of 80 μg/ml CM-GAPD; Curve C, inactivation of GAPD at 80 μg/ml. The experiments were carried out at 45 °C in 0.05 ionic strength tris/HCl buffer, pH 8.5, containing 5×10^{-3} M 2-mercaptoethanol

We observed that the ionic composition of buffers also influenced the heat stability of GAPD; for example high concentration of chloride, sulfate or phosphate decreased the rate constants of heat inactivation. Keleti and Szegvári (1972) have studied the heat inactivation of GAPD *holoenzyme* in 0.1 M glycine/NaOH buffer, pH 8.5, containing 0.03 M 2-mercaptoethanol and 0.1 M NaCl. To be

able to compare our results with theirs we examined also in this medium the heat stability of GAPD *apoenzyme*. We found that inactivation proceeded very much like in 0.05 ionic strength tris/HCl buffer, pH 8.5, containing 2-mercaptoethanol, between 20 and 40 °C, only at 45 °C was inactivation slower by a factor of 0.8 – 0.9, presumably due to the effect of chloride ion.

5. *Hybrid formation of native and carboxymethylated subunits.* Kinetic measurements of heat inactivation indicated that subunits of the active enzyme can associate with those of carboxymethylated inactive enzyme (CM – GAPD) to form hybrid dimers (Fig. 5). The figure shows that inactivation of GAPD at 1 μg/ml concentration is slowed down if inactive CM – GAPD in a higher concentration (80 μg/ml) is also present. This indicates that the monomer of the active enzyme can form a hybrid with the carboxymethylated monomer, since the large amount of CM-monomers shifts the dissociation equilibrium towards association, i.e. decreases the concentration of native monomers. Hybrid formation of GAPD was shown by others too (Spotorno, Hollaway, 1970; Meighen, Schachman, 1970; Ovádi et al., 1971).

In conclusion, the heat stability of GAPD depends on enzyme concentration. This concentration dependence below 0.1 mg/ml can be explained by the predominance of the dimer–monomer dissociation equilibrium of the protein molecule.

Appendix

The values of the dissociation constant of dimer–monomer equilibrium and the rate constants of inactivation of dimeric and monomeric forms were obtained in the following way. Assuming that at enzyme concentrations below 50 μg/ml the dimer–monomer dissociation plays a dominant role and the concentration of tetrameric form is negligible, the rate of inactivation can be described by the following equation

$$k_{\text{measured}}\,[E] = k_{\text{monomer}}\frac{[m]}{4} + k_{\text{dimer}}\frac{[d]}{2} \tag{4}$$

where $[E]$ = total concentration of active enzyme (tetramer),
$[m]$ = molar concentration of monomeric form in the active enzyme,
$[d]$ = molar concentration of dimeric form in the active enzyme,
k_{measured} = first order rate constant calculated from the initial part of the reaction,
k_{monomer} = first order rate constant of inactivation of the monomeric form,
k_{dimer} = first order rate constant of inactivation of the dimeric form.

Rearranging equation (4) and using the equation of dimer–monomer dissociation equilibrium we obtain:

$$\frac{1}{k_{\text{measured}} - k_{\text{dimer}}} = \frac{1}{k_{\text{monomer}} - k_{\text{dimer}}} + \frac{2}{K_d/k_{\text{monomer}} - k_{\text{dimer}}/}[m] \tag{5}$$

where K_d = dissociation constant of dimer–monomer equilibrium. We selected the values of K_d and k_{dimer} by trial and error which gave a straight line in the plot $\frac{1}{k_{\text{measured}} - k_{\text{dimer}}}$ vs. $[m]$ with intercept on the ordinate $\frac{1}{k_{\text{monomer}} - k_{\text{dimer}}}$ and on the abscissa $-\frac{K_d}{2}$ (cf. equation (5)). We found agreement with the experimental data only if the value of k_{dimer} was chosen less than 10 % of k_{monomer}. Therefore in the calculations we neglected the value of k_{dimer}. The K_d and k_{monomer} values at different temperatures which give the best fit with experimental data are shown in Table 1.

Thanks are due to Prof. F. B. Straub for continuous interest in this work. The authors also wish to express their thanks to Dr G. Szabolcsi and Mr. P. Arányi for helpful discussions and to Miss É. Kneisz for skilful technical assistance.

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Heat Denaturation of D-glyceraldehyde-3-phosphate Dehydrogenase Holoenzyme

(Short Communication)

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The conformational stability of a protein depends on the pH, temperature and composition of the medium and on the steric structure of the protein itself and it is reflected in the activation energy of denaturation. The aim of this work was to determine the rate constant and activation energy of heat denaturation of D-glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating) – EC 1.2.1.12 – containing the firmly bound NAD and the effect of pH and protein concentration on these parameters.

The heat denaturation of the enzyme, as determined by measuring the loss of enzymic activity in glycine buffer containing 0.03 M 2-mercaptoethanol, follows first order kinetics throughout the pH-range (pH 5.2 to 9.2), concentration range (0.030 to 5.0 mg/ml) and the temperature range (37 to 60 °C) examined even up to about 90% loss of initial activity. Some progress curves are presented in Fig. 1.

These results permit the assumption that the heat denaturation of glyceraldehyde-3-phosphate dehydrogenase holoenzyme follows the all-in-one mechanism (Keleti, 1971). However, in tris-HCl buffer, ionic strength 0.05, downward concave progress curves of inactivation were observed in the semilogarithmic plots after 70–80% loss of activity in agreement with the data obtained with the apoenzyme (Vas, Boross, 1972).

The first order rate constant of heat inactivation has a pH-minimum. This minimum is between pH 7.5 and 8.0 at 1 mg/ml protein concentration and 50 °C (Fig. 2).

The Arrhenius plots of heat inactivation at pH 7.5 to 8.0 in glycine buffer containing 0.03 M 2-mercaptoethanol, with protein solutions of 0.030 to 0.100 mg/ml (between 37 and 47°C), of 0.125 to 1.0 mg/ml (between 43 and 55 °C) and of 2.0 to 5.0 mg/ml (between 47 and 60 °C) gave straight lines and indicated an average value of activation energy of about 100 ± 15 kcal/mole. In tris-HCl buffer (0.05 ionic strength) the same activation energy was measured (5 mg/ml holoenzyme in the presence of 2-mercaptoethanol, between 50 and 57 °C). Even charcoal treated (NAD-free) apoenzyme gave the same activation energy at 1 mg/ml concentration (in 0.1 M glycine buffer, containing 0.1 M NaCl and 0.03 M 2-mercaptoethanol, between 43 and 55 °C).

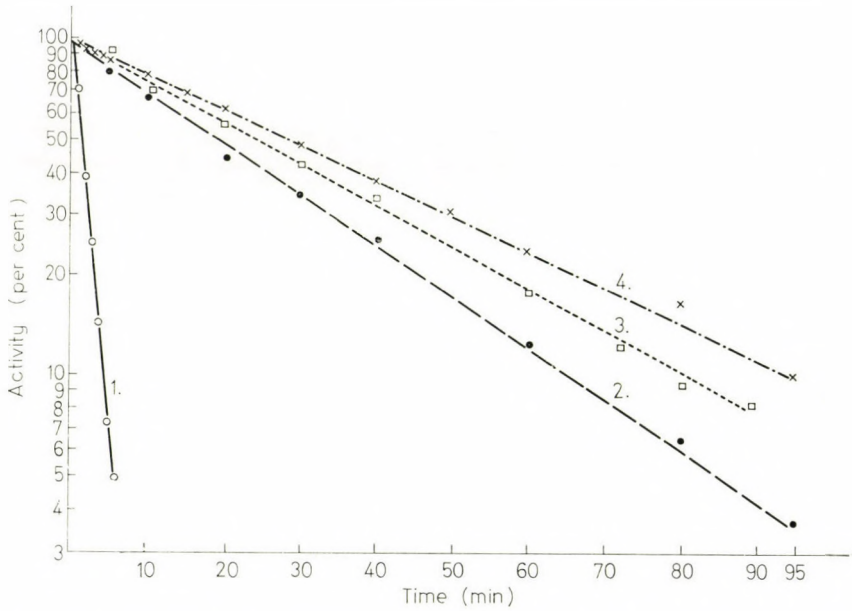

Fig. 1. Heat inactivation of D-glyceraldehyde-3-phosphate dehydrogenase under various conditions. Four times recrystallized swine muscle glyceraldehyde-3-phosphate dehydrogenase (Elődi, Szörényi, 1956) dissolved in 0.1 M glycine buffer, containing 0.1 M NaCl and 0.03 M 2-mercaptoethanol, pH 8.5 at 18 °C (which corresponds to pH 7.7 at 50 °C) was diluted with the same buffer heated to the temperature of the experiment. At the times indicated *aliquots* were cooled to 0 °C and diluted: a) with the same buffer to obtain the necessary concentration in the cuvette for enzymic activity assay, and b) with 0.1 M NaOH for the determination of protein concentration by using the molar extinction coefficient 145 000 for the holoenzyme at 280 nm. The enzymic activity was measured in a Hilger UVISPEK spectrophotometer at 340 nm in silica cells of 1 cm light path in the following assay mixture: NAD 2×10^{-3} M, phosphate 1×10^{-2} M, D-glyceraldehyde-3-phosphate 2×10^{-3} M, enzyme 7.15×10^{-9} M in 0.1 M glycine buffer, pH 8.5, at 18 °C. The figure shows the loss in per cent of specific enzyme activity in semilogarithmic presentation as a function of time. 1: 0.125 mg/ml protein, at 53 °C; 2: 1.0 mg/ml protein, at 55 °C; 3: 0.030 mg/ml protein, at 47 °C; 4: 5.0 mg/ml protein, at 57 °C

The enzyme in solution forms an equilibrium mixture of tetrameric, dimeric and monomeric species (Hoagland, Teller, 1969; Lakatos et al., 1972; Ovádi et al., 1971), the firmly bound NAD's have different dissociation constants (Koshland, Neet, 1968; De Vijlder, Slater, 1968) and the apparent first order rate constant of heat inactivation of the apoenzyme changes with protein concentration (Vas, Boross, 1972). Considering the aforementioned data one would expect the activation energy to change with protein concentration and a deviation from linearity of the kinetics of denaturation and of the Arrhenius plots. However, we were unable to demonstrate any of the above phenomena in the concentration and temperature ranges used.

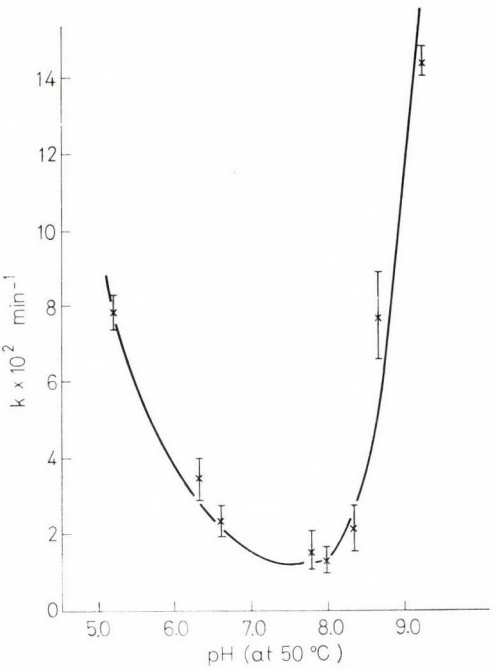

Fig. 2. pH-dependence of first order rate constant of heat inactivation of glyceraldehyde-3-phosphate dehydrogenase. The first order rate constants were determined as in Fig. 1, at 1 mg/ml protein concentration and 50 °C. The pH was measured with a Radelkisz blood pH-meter at 50 °C at the beginning and at the end of heat exposure and only those experiments were accepted where the difference in pH did not exceed ± 0.2 units

The rate constants of heat inactivation of the holoenzyme were only slightly influenced by protein concentration. This change may partially be due to the change in the saturation by NAD, since it is known that the binding of NAD alters the steric structure of the protein and it becomes more stable (Előd i, Szabolcsi, 1959; Szabolcsi 1958; Listowsky et al., 1965; Závodszky et al., 1966). Indeed, we have shown that the saturation of the enzyme in dilute solution by excess NAD decreases the first order rate constant of inactivation by a factor of about 0.7, as compared to the "holoenzyme", which in dilute solution (0.030 to 0.125 mg/ml) contains about 2 moles of firmly bound NAD per mole of protein. A similar protective effect was found by increasing the SO_4^{2-} concentration of dilute enzyme solutions.

The dimeric form of the enzyme is probably less stable than the tetramer as it was shown in experiments with enzyme-ATP complex (Ovádi et al., 1971). However, at higher temperatures higher average molecular weights can be determined (Závodszky, 1965) and the dissociation of the enzyme into subunits caused by ATP or salts is promoted by low but reversed by high temperature (Constantinides, Deal 1969, 1970; Nagradova, Güseva, 1971). These data suggest

that higher temperatures favour the association of the subunits. Further data suggest that NAD may also displace the equilibrium towards the associated form (unpublished results).

If the assumption is true that under the conditions used the equilibrium is greatly shifted towards the tetrameric form, the observed denaturation is that of the tetrameric form of glyceraldehyde-3-phosphate dehydrogenase. This notion is supported by the higher activation energy of heat inactivation of the holoenzyme at any protein concentration and of the apoenzyme at high concentration and high temperature (about 100 kcal/mole) as compared to that of the apoenzyme dissociated into monomers (about 30 kcal/mole, see Vas, Boross, 1972).

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Spin Labelling of D-glyceraldehyde-3-phosphate Dehydrogenase with Specific Reagents

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Blocking of D-glyceraldehyde-3-phosphate dehydrogenase with spin-labelled iodoacetate or iodoacetamide results in a spectrum which can be regarded as a sum of ESR signals of rotationally slightly hindered and immobilized radicals. In the apoenzyme whose reactive SH-group is blocked with spin-labelled iodoacetate the proportion of immobilized radicals increases on the addition of NAD. The inhibitory effect of non-labelled iodoacetate differs from that of the spin-labelled reagent. In contrast to the non-labelled alkylating agents, the spin-labelled compounds induce structural changes in the enzyme and consequently SH-groups other that the reactive ones are also blocked. In case of spin-labelled alkylating reagents the sulfhydryls that reacted after the reactive ones belong to Cys-153. It seems reasonable to assume that there is a cleft on the surface of the protein near the reactive SH, the width of which is comparable to the maximal diameter of spin-labelled reagent, and that the peptide sequence between residues Cys-149 and Cys-153 is of non-helical structure.

Introduction

The method of spin-labelling can be equally used for the detection of local conformational changes of molecules and supramolecular structures (McConnell, Hamilton, 1968; Hamilton, McConnell, 1970; McConnell, McFarland, 1970). In a previous work we studied the formation of a $g \sim 2.03$ paramagnetic centre, and its effects, in GAPD* as a model protein. This centre was found to be bound to the histidyl and SH-groups of GAPD and showed competitive inhibition with GAP substrate (Elek, Keleti, 1971). In the present work our aim was to block the already well-characterized reactive side chains of the enzyme with specific reagents labelled with free radicals, and to monitor the conformational changes of the protein by the aid of ESR spectrum. The conformational changes of the enzyme have already been studied by several authors by different methods (Előd, Szabolcsi, 1959; Listowsky et al., 1965; Havsteen, 1965; Bolotina et al., 1966; Závodszky et al., 1966). Thus the data obtained from spin-label experiments may contribute to the picture of the structure of the enzyme and, furthermore, may serve as a control of the usefulness of the method.

* *Abbreviations:* DEP = diethylpyrocarbonate; ESR = electron spin resonance; GAPD = D-glyceraldehyde-3-phosphate dehydrogenase; GAP = D-glyceraldehyde-3-phosphate; pMB = p-(hydroxy)-mercuribenzoate; R = iminoxyl radical.

Materials and methods

GAPD holoenzyme was prepared from pig muscle and four times recrystallized according to the method of Elődi and Szörényi (1956). GAP was prepared from fructose-1,6-diphosphate (Szewczuk et al. 1961). NAD was purchased from Reanal, DEP from Schuchardt, mono-iodoacetate and iodoacetamide from Light and pMB from British Drug Houses Ltd. All other chemicals were commercial preparations of reagent grade.

The reagents spin-labelled with nitroxide-radical were prepared from 2,2,6,6-tetramethyl-4-oxopiperidine-1-oxyl; 2,2,6,6-tetramethyl-4-oxypiperidine-1-oxyl or 2,2,6,6-tetramethyl-4-aminopiperidine-1-oxyl. The first one was synthetized by us, the last two were kindly supplied by Dr E. G. Rozantzev. The spin-labelled reagents were prepared by Drs A. B. Shapiro and V. I. Suskina according to the procedures summarized in Table 1.

Spin labelling: The reagent was dissolved in a few drops of the solvent (cf. Table 1), then after dilution with 0.1 M phosphate buffer, pH 7.0, it was added to a 40 to 50 mg/ml enzyme solution. After 18 to 24 hours of incubation at 0 °C the mixtures were dialyzed and the solution was gel-filtered on a Sephadex G–50 column (2×30 cm) equilibrated with the same buffer, in order to remove unbound spin-labelled compounds. The spectra of spin-labelled protein solutions (10^{-4} M) were registered in an IHF-2, JES-P-10 or JES-ME-3X spectrometer. The distance of spectral bands was determined on the basis of proton resonance calibration of the magnetic field (Hedvig, Zentai, 1969; Mohos, Hummel, 1972).

To estimate the average distance between spin-labells on the protein molecule, the solution was diluted with an equal volume of glycerol, frozen in liquid nitrogen, and the d_1/d ratio of the spectrum was read from the diagram (Fig. 1). The average distance between radicals is thus obtained in Å (Kokorin et al., 1972).

If the amount of radicals bound to protein had to be determined, the spectrum of the sample diluted with glycerol was registered together with a $Mn^{2+}-MgO$ control, then the measured signal amplitude was normalized with the Mn band. The signal amplitude was compared with that of the spectra of iminoxyl radical of known concentration, dissolved in glycerol and cooled to −196 °C. Correlation time was calculated as described by Buchachenko and Vasserman (1967).

Enzyme activity was assayed as described earlier (Keleti, Batke, 1965).

The number of SH-groups was determined by titration with pMB (Boyer, 1954), *histidines* were measured by the aid of DEP (Ovádi et al., 1967).

Tightly bound *NAD was removed* from GAPD, i.e. apoenzyme was prepared, by treatment with charcoal (Velick, 1953).

Carboxymethylation. The protein already blocked with spin-labelled reagent was carboxymethylated with ^{14}C-bromoacetate in 8 M urea as described by Harris and Perham (1965). Tryptic digestion was carried out in 0.1% ammonium bicarbonate solution at pH 8.2 at 37 °C for 2.5 hours with 1/30 part trypsin (Calbiochem, essentially free from chymotrypsin). Hydrolysis was stopped by boiling. The hydrolysate was freeze-dried.

Table 1

Compounds used for the spin-labelling of GAPD

The compound (1 mg) was dissolved in the solvent indicated, and after dilution with buffer it was incubated with about 2 ml of a 50 mg/ml enzyme solution for 24 hours, then filtered. The solution was passed through a Sephadex G-50 column and then the ESR spectrum was registered. R stands for the iminoxyl radical coupled to the compound; in case of compounds 1, 3, 4, 10 it is 2,2,6,6-tetramethyl-4-oxypiperidine-1-oxyl, in case of compounds 2, 5, 6, 9 it is 2,2,6,6-tetramethyl-4-aminopiperidine-1-oxyl and in case of compound 7 it is 2,2,6,6-tetramethyl-4-oxopiperidine-1-oxyl

No.	Spin-labelled reagent		Comment	Specificity	Reference to the preparation of reagent	ESR signal on GAPD after gel-filtration
	Name	Solvent and temperature and pH of labelling				
1	monoiodoacetic acid-R	alcohol, 0 °	Hydrolyzes at alkaline pH	SH	Rozantzev (1970) Kohanov (1971)	+
2	iodoacet-amide-R	alcohol, 0 °	Does not precipitate on dilution with water	SH	Rozantzev (1970) Rozantzev, Kohanov(1966) McConnell, Hamilton (1968)	+
3	pMB-R	dimethyl sulfoxide, 0 °	Immediately precipitates on dilution with water	SH	Shapiro (1971)	+
4	Ethylene imine-R	water, 0 °	Strongly hygroscopic, poorly soluble in water	SH and imida-zole	Shapiro et al. (1969)	+
5	cyanuric chloride-R	acetone, room tem-perature pH 6	Forms suspen-sion on dilution with water	pH 6: imida-zole pH 8: lysine	Lichtenstein, Bobodjanov (1969)	—
6	butyric acid-R	alcohol, 0 °	Can be used as a spin-probe	hydrophobic areas	Rozantzev (1964) Goldfield et al. (1970)	—
7	R	water, room temperature	Strongly hygroscopic	—	Rozantzev (1970)	—
8	ethyl iodide-R	alcohol, 0 °	Does not precipitate on dilution with water	—	Rozantzev et al. (1970) Ivanov (1970)	—
9	tertiary amine-R	water, 0 ° pH 7.6	Fairly soluble in water	—	Suskina (1971)	—
10	acrylic acid-R	alcohol, 0 ° pH 7.6	Precipitates on dilution with water	—	Suskina (1971)	—

Electrophoresis was performed in pyridine–acetic acid buffer, pH 6.5 (pyridine–acetic acid–water, 90 : 4: 400) and in acetic acid–formic acid buffer, pH 1.9 (acetic acid–formic acid–water, 8 : 2 : 90), at 50 V/cm and 80 V/cm voltage gradient, respectively. The position of radioactive peptides was detected by radioautography.

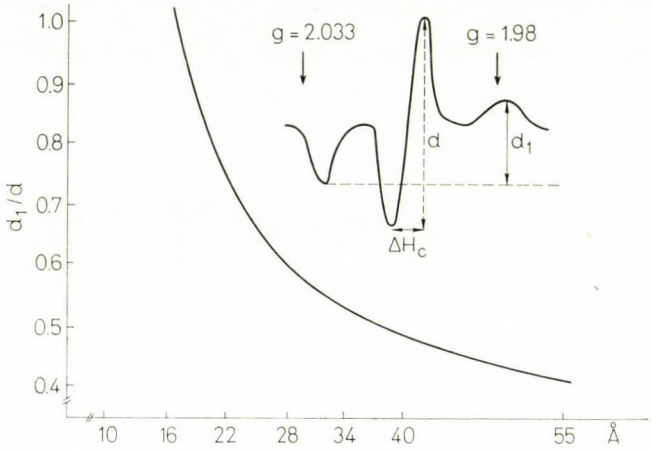

Fig. 1. Estimation of the distance between isotropically distributed nitroxide radicals. The solution of the enzyme sample spin-labelled with nitroxide radicals was diluted with an equal volume of glycerol. The solution was cooled to the temperature of liquid nitrogen. In the glass-like gel thus formed there is no movement of the radicals, therefore in the spectrum of nearby nitroxide radicals the broadening effect of dipole-dipole interaction can be detected (cf. Hedvig, Zentai, 1969). Above all the medium band of the triplet is broadened. Therefore in the spectrum of the immobilized nitroxide radical (cf. the insert) the parameters d and d_1 indicate the distance between pairs of radicals. The calibration curve gives the relationship between the ratio of these parameters (ordinate) and the distance of spin-labels in Å (abscissa). ΔH_c is the width of the medium band. If the radicals are not randomly distributed but in pairs, the distance read from the graph should be multiplied by 0.7. The nomogram was obtained by analyzing solutions of different concentrations of bi- and monoradicals (Kokorin et al., 1972)

Results

The data of spin-labelled compounds are summarized in Table 1.

After passing through a Sephadex column once, only enzyme solutions treated with compounds 1, 2, 3 and 4 gave ESR spectra. All four compounds are SH-reagents. Although iminoxyl groups may also react with SH-groups (Buckman, 1970), compound 7 did not inhibit the enzyme activity of GAPD.

The pMB-R derivative was very poorly soluble, even more so than pMB derivatives prepared by others (Boyens, McConnell, 1966; Lichtenstein et al., 1968), therefore it could not be used.

Although there are extensive hydrophobic areas in GAPD, gel filtration through a Sephadex column removed spin-probes* (6, 7, 9) that merely adsorbed to the protein just as well as cyanuric chloride-R (5)** ethyliodide-R (8) or the spin-labelled acryl derivative (10).

Of the compounds that were bound to the protein, only those were used which were fairly soluble and data were available about their non-labelled forms.

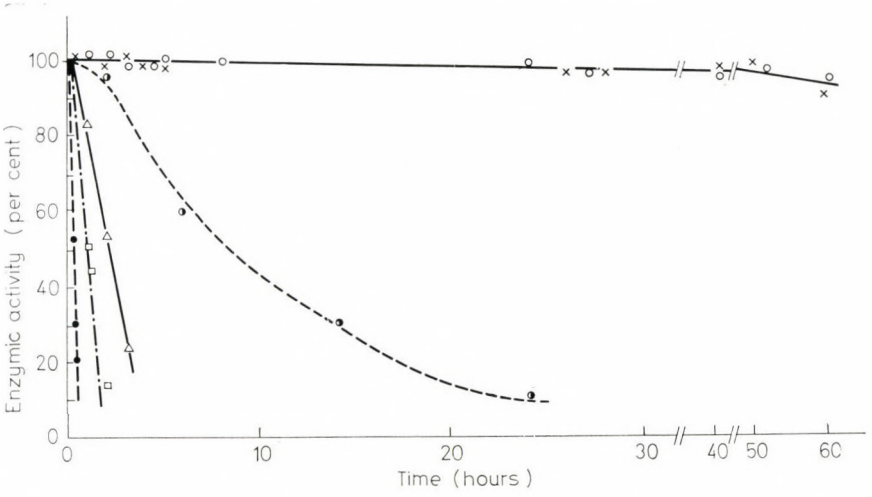

Fig. 2. Effect of spin-labelled reagents on the activity of glyceraldehyde-3-phosphate dehydrogenase. The enzyme solution (28—35 mg/ml) in 0.1 M phosphate buffer, pH 7.0, was mixed with a few drops of concentrated reagent so that the mixture should contain 8 moles of reagent per mole of enzyme. The protein was incubated in the case of cyanuric chloride at room temperature, in the case of SH-reagents at 0 °. At the times indicated samples were withdrawn and after appropriate dilution the enzyme activity was assayed as described in Methods. × = control enzyme, ○ = cyanuric chloride-R + holoenzyme. The mixture also contained 10^{-2} M mercaptoethanol. • = iodoacetate-R (4 equivalents) + apoenzyme, △ = iodoacetate-R + holoenzyme, □ = iodoacetamide-R + apoenzyme, ◑ = iodoacetamide-R + holoenzyme

Fig. 2 shows the time course of inactivation of the enzyme in the presence of cyanuric chloride-R, iodoacetate-R and iodoacetamide-R, respectively.

Cyanuric chloride-R did not affect the activity of the enzyme, after treatment 24 histidines could be titrated with DEP, just as in the native untreated enzyme (cf. Ovádi, Keleti, 1969). Thus the unchanged enzyme activity and number of

* Spin-labels which are not covalently bound but merely fixed to the structure due to their hydrophobic nature or lipid solubility by adsorption, are referred to as spin probes (Hubbell, McConnell, 1969; Goldfield et al., 1970).

** Cyanuric chloride-R has been successfully used by Lichtenstein et al. (1969, 1970) for spin-labelling the imidazole groups of several proteins.

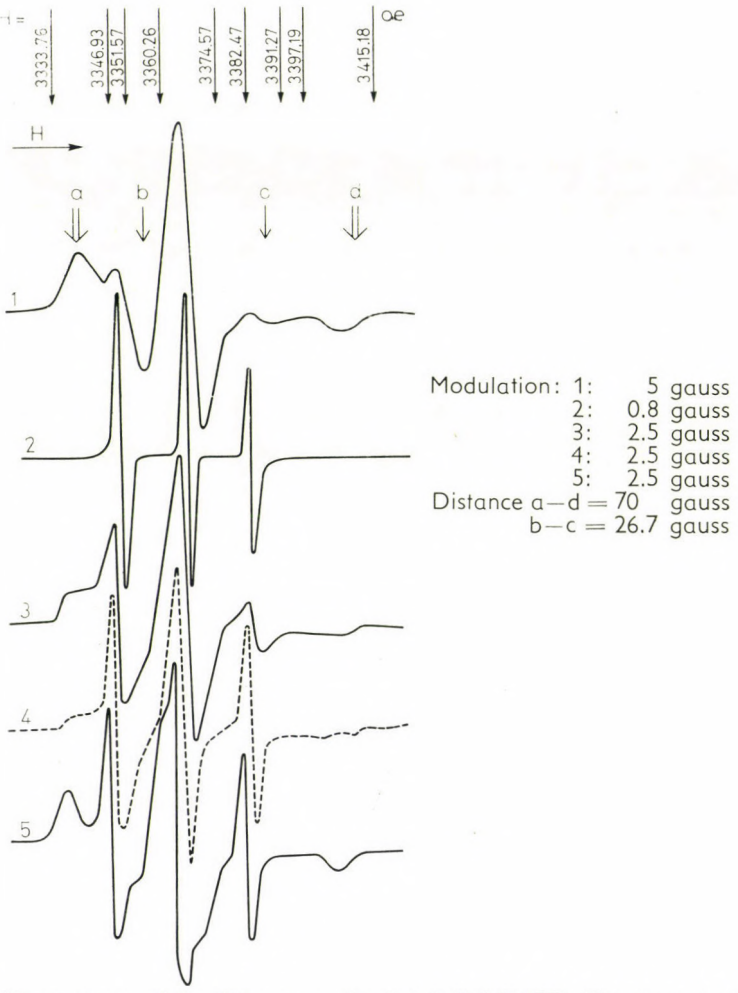

Fig. 3. ESR spectrum of the SH-groups of spin-labelled GAPD. The enzyme solution (50 mg/ml) was incubated with SH-blocking reagents (spin labelled) for 24 hours. After passing the solution through a Sephadex G-50 column (1.5 × 15 cm), the ESR spectra were registered. 1. Four mole-equivalents of iodoacetamide-R were added to the apoenzyme. The radical bound to the reactive SH-group is immobilized. The spectrum does not change on the saturation of the labelled enzyme with NAD. 2. The above enzyme solution was saturated with urea. The picture is characteristic of a radical which is slightly hindered. 3. The apoenzyme was incubated with 10 equivalents of iodoacetamide-R. The spectrum is composed of the two previous spectra. The spin-label is partly immobilized, partly nearly freely moving. 4. The apoenzyme was incubated with 4 equivalents of iodoacetate-R. The spin-label bound to the reactive SH-groups is partly freely moving, partly immobilized. 5. To the previous solution 1/10 volume of 2% NAD was added. After 10 min incubation a part of the freely moving label becomes immobilized. The spectrum is the same if the holoenzyme is labelled with iodoacetate-R. Incubation with 8 to 12 equivalents of iodoacetate-R only increases the intensity of the spectrum, the pattern is a superposition of spectra of almost freely moving and immobilized radicals. The figures on the lines indicate the intensity of the magnetic field in gauss (oe)

histidine residues, as well as the lack of ESR signal, unequivocally indicate that the histidyl residues of GAPD did not react with cyanuric chloride-R.

As shown in Fig. 2, both iodoacetamide-R and iodoacetate-R inactivate the apoenzyme faster than the holoenzyme.

Some characteristic spectra are shown in Fig. 3. From the spectra conclusions can be drawn as to the motility of the radical. Motility can be quantitatively characterized by the correlation time, τ (cf. Hedvig, Zentai, 1969); the smaller the value of τ the greater the freedom of motility. The spectrum of iminoxyl radical consists of three uniform bands in solutions of low viscosity. The width of triplet is 30–31 gauss. This spectrum is similar to that of the enzyme labelled with iodoacetamide-R, in urea solution (spectrum 2) but the unequal height of bands points to larger correlation time, $\tau = 5.8 \times 10^{-10}$ sec. If the movement of the radical is sterically more strongly hindered and therefore slower, the anisotropy of the magnetic resonance absorption is not leveled. The distance marked by arrows *a* and *d* is about 70 gauss, i.e. much greater than the distance between the two extreme bands of the spectrum of solution 2. Those radicals absorb at a distance the *z* axis of which is nearly parallel with the magnetic field, i.e. the coupling constant of direction *z* prevailed (Hubbel, McConnell, 1969). The "immobilized" iodoacetamide-R bound to the enzyme gives such a broad spectrum (cf. spectrum 1). In case of such strongly fixed radicals the approximate value of correlation time can be estimated by comparing our spectra with simulated spectra obtained for different correlation times (Lazarev, Strukov, 1971). In this way $\tau = 2-5 \times 10^{-8}$ was obtained from spectrum 1. If more iodoacetamide-R was added to the enzyme than required for the blocking of the four reactive SH-groups per molecule, the spectrum of more freely-moving radicals also appeared, i.e. the picture is composed of spectra 1 and 2 (spectrum 3). Iodoacetate-R bound to the 4 reactive thiols already yields a spectrum characteristic of a greater freedom of movement (spectrum 4). If the apoenzyme was labelled with 4 mole-equivalents of iodoacetate-R, the addition of NAD increased the contribution of immobilized radicals to the spectrum (spectrum 5). In contrast, the addition of NAD to the apoenzyme blocked with 4 mole-equivalents of iodoacetamide-R did not change the spectrum. On the addition of 8 mole-equivalents of iodoacetate-R to the enzyme, the intensity of the spectrum further increases after the inactivation of the enzyme. From this finding it was concluded that beside the reactive thiols further SH-groups also become labelled. To test the validity of this assumption, the number of SH-groups remaining after labelling was determined (Table 2).

After incubation with 16 mole-equivalents of iodoacetate-R for 24 hours no SH-groups could be detected on the enzyme. On the other hand, if the 4 reactive thiols were blocked with non-labelled iodoacetate, the enzyme did not bind any more iodoacetate-R. This enzyme sample contained 10 to 13 free SH-groups per molecule even after prolonged incubation with 16 mole-equivalents of iodoacetate-R. Thus the spin-labelling of SH-groups other than the reactive ones could only be achieved if the reactive thiols were also blocked with spin-

Table 2

Changes in the number of SH-groups of GAPD after treatment with spin-labelled iodoacetate

The number of SH-groups was determined by the method of Boyer (1954). 1. Untreated enzyme; 2. Enzyme at a concentration of 10 mg/ml was incubated with 16 mole-equivalents of iodoacetate-R. After inactivation was complete, half of the sample was gel-filtered on a Sephadex G-50 column and the number of SH-groups was determined; 3. The other half of the sample was further incubated in the refrigerator for 24 hours. The precipitated enzyme was then dissolved in urea solution and the SH-groups were titrated in this urea solution. 4. Another enzyme solution was incubated with 6 mole-equivalents of non-spin labelled iodoacetate for 6 hours, then gel-filtered, and incubation was continued in the presence of 16 mole-equivalents of spin-labelled iodoacetate-R for 16 hours. At the end the sample was gel-filtered and the number of SH-groups was determined

No. of sample	Number of free SH-groups per molecule
1	15—16
2	11—13
3	0
4	10—13

labelled iodoacetate-R. It appears that if the reactive thiols are blocked with non-labelled iodoacetate, a conformational change which would result in the exposure of further SH-groups does not take place. On the other hand, the binding of spin-labelled iodoacetate leads to such steric alterations.

In the primary structure of the protein at a distance of 4 residues from the reactive cysteine there is another SH-group (Harris et al., 1963). If the second iodoacetate-R bound per subunit is linked at this position, then it is very near to the free radical bound to the reactive thiol and thus may interact with it. By the aid of the method for the determination of the distance between nearby radicals (Kokorin et al., 1972) we compared the samples incubated with 4, 8, 12 and 16* mole-equivalents of iodoacetate-R (Table 3).

The d_1/d ratio of ESR spectrum (Fig. 1) only increased with samples incubated in the presence of 16 mole-equivalents of iodoacetate-R, which indicates the interactions of spins. If 8 mole-equivalents of reagent were added the radicals were distributed in the solution not in a random manner but by pairs and the distance evaluated had to be multiplied by 0.7. Therefore in this case the distance between two radicals located on the same subunit is not 50 Å, as would seem from Fig. 1, but 33 to 35 Å. This method, however, gives exact results only for the ascending part of the calibration curve, i.e. for small distances (<28 Å).

We examined by the fingerprint method that in the holoenzyme labelled with 4 or 8 mole-equivalents of iodoacetate-R or in the apoenzyme labelled with

* In the last two cases the enzyme precipitated, therefore ions could only be removed by dialysis.

Table 3

Average distance of free electrons on the enzyme molecule spin-labelled on the SH-groups

The enzyme solution spin-labelled with iodoacetate-R was diluted with an equal volume of glycerol and the ESR spectrum was registered at the temperature of liquid nitrogen. From the d_1/d ratio of the spectrum the distance was obtained by the aid of Fig. 1 (Kokorin et al., 1972)

Number of spin-labelled SH-groups per enzyme molecule	Average d_1/d ratio (4 experiments)	Average distance of electrons of the free radicals, Å
4	0.404 ± 0.026	$>53(>37)$*
8	0.406 ± 0.018	$>50(>35)$*
12	0.445 ± 0.029	48(33)*
16	0.527 ± 0.065	34(24)*

* Since the interacting radicals are of non-random distribution but occur in pairs, the distance read from the graph of Fig. 1 should be multiplied by 0.7. These figures are shown in brackets

the same amounts of iodoacetamide-R or iodoacetate-R which sulfhydryl groups were blocked. To this end the spin-labelled samples were carboxymethylated in 8 M urea with ^{14}C-bromoacetate, i.e. the SH-groups that did not react with the spin-labelled compounds were modified. After carboxymethylation the protein was subject to tryptic hydrolysis, and the hydrolysate was fingerprinted and the

Table 4

^{14}C-labelled S-carboxymethyl peptides found in the tryptic hydrolysate of spin-labelled and control GAPD

The peptides were identified by two-dimensional electrophoresis followed by radioautography

Sample	Part of the sequence*		
	143–159**	232–248	269–306
Holoenzyme	+	+	+
Holoenzyme treated with 8 mole-equivalents of iodoacetate-R	–	+	+
Apoenzyme	+	+	+
Apoenzyme treated with 4 mole-equivalents of iodoacetamide-R	–***	+	+
Apoenzyme treated with 8 mole-equivalents of iodoacetate-R	–	+	+

* Numbered on the basis of sequence published by Harris and Perham (1968)

** The tryptic peptide contains two cysteines, one of which (Cys-149) carried a reactive SH-group (Harris et al., 1963)

*** For explanation cf. the text

radioactive peptides were localized by radioautography as described by Harris and Perham (1965). In the hydrolysate of sample labelled with 8 mole-equivalents of reagent there was no radioactive component corresponding to the position of the peptide containing the reactive thiol, whereas all hydrolysates comprised the ^{14}C-carboxymethylated tryptic peptides corresponding to portions 232–248 and 269–306 of the sequence (Table 4).

In the sample containing 1 spin-labelled radical per subunit the bulk of radioactivity could be detected in the neutral fraction after tryptic hydrolysis following carboxymethylation, which is in agreement with the fact that peptide 143–159 contains in addition to the reactive thiol another cysteine too, which is then labeled during the course of full carboxymethylation with ^{14}C-bromoacetate. On electrophoresis at pH 6.5 this peptide is found in the neutral fraction, since only one of the two SH-groups carries the negatively charged carboxymethyl group.

Discussion

The blocking of reactive functional groups of enzymes is a classical method in the investigation of structure and function. However, the properties of spin-labelled reagents differ from those of the traditional reagents.

In the present study iodoacetate-R and iodoacetamide-R proved to be applicable with advantage. In the presence of these reagents GAPD is inactivated, with different rates, and there are also differences in the ESR spectrum of the two kinds of spin-labelled enzyme. It is known that GAPD is inactivated by non-labelled iodoacetate and iodoacetamide in different manners. The holoenzyme is inactivated faster with iodoacetate than is the apoenzyme (Racker, Krimsky, 1958). This differential reactivity has been attributed to a salt linkage between the carboxyl group and pyridinium ion. This bond would orient iodoacetate so as to facilitate the carboxymethylation of the reactive SH-group (Cseke, Boross, 1970). In contrast to the above scheme, iodoacetate-R inactivates the apoenzyme more rapidly. This might be due to the circumstance that the salt linkage cannot be formed any longer as the carboxyl group is involved in the ester bond that stabilizes the radical. NAD acts merely as a sterically hindering component and decreases the rate of binding. Iodoacetate-R bound to the apoenzyme is actually hindered in its rotation by subsequently added NAD, which is directly shown by the ESR spectrum.

It has been claimed that NAD forms a charge-transfer complex with the reactive SH-group (Boross, Cseke, 1967), therefore these two groups should be in juxtaposition. It may be inferred that NAD probably interferes locally with the movement of the spin-label and not indirectly by altering the steric structure of the enzyme, although this possibility cannot be excluded.

Balthasar (1971) also studied the ESR spectrum of GAPD spin-labelled on the SH-groups. It was concluded from these investigations that the observed ESR signal, similarly to our case, was the summation of spectra of free, sterically

hindered and immobilized radicals. In contrast to our findings, on the addition of NAD an increase in the intensity of the free component could be detected. This apparent contradiction may well be explained by the facts that in the above mentioned studies the enzyme was isolated from yeast and rabbit muscle and the geometry of the spin-labelled reagent considerably differed from ours as it contained a five-membered ring and it was much longer than iodoacetate-R used by us. It is known from the literature that the number of atoms of the ring affects the spin-label (Tylor et al., 1969), and so does the length of the carbon chain (Lichtenstein et al., 1970).

Non-labelled iodoacetamide alkylates the SH-groups of the apoenzyme faster than those of the holoenzyme (Cseke, Boross, 1970). The same holds for iodoacetamide-R.

In the case of iodoacetate-R it could be raised that the part of the spectrum attributed to free-moving radicals was due to hydrolyzed radicals, i.e. to radicals where R was split from the iodoacetate moiety after gel-filtration. However, on the addition of NAD to the spin-labelled apoenzyme a part of the free-moving radicals was converted into tightly bound form. This finding excludes possible hydrolysis. Thus the spectrum of the enzyme labelled on the reactive thiols with iodoacetate-R is composed of two spectra: those of the nearly free and of the immobilized radicals. On the other hand, the enzyme labelled with iodoacetamide-R only exhibits the spectrum of immobilized radicals, and the pattern characteristic of more freedom of movement only appears if further SH-groups are also labelled. This immobilized spectrum greatly resembles those observed with membranes, erythrocytes and nerve fibres (Hubbell, McConnell, 1969). The shape of the spectrum can be reconciled with a model in which only the axial rotation, or the random movement within a cone, of the radical is allowed (Jost et al., 1971). This movement can be visualized as follows: the radical fixed at one end rotates in a cylindrical cavity, i.e. immobilization is anisotropic. If the free radical can also be appreciably moved sideways (angular deviation), anisotropic rotation may turn into isotropic. The smaller are the movements sideways, the more approaches the movement anisotropic rotation, and the farther are then located maxima *a* and *d* on spectrum 1 in Fig. 3 from each other. Thus from the distance of the two extreme bands of the spectrum of immobilized radical the angle of the cleft in which the iodoacetamide-R is positioned can be estimated (Hubbell, McConnell, 1969). If this model also holds in the case of GAPD then angular deviation is practically 0. Accordingly, the width of the cleft equals the maximal width of the spin-labelled compound that is accommodated in the cleft.

Non-labelled iodoacetate and iodoacetamide are only bound to the reactive thiol (Cys-149) in the native enzyme. However, the titration of SH-groups and the fingerprint analysis of the native enzyme treated with spin-labelled reagents indicate that further SH-groups became blocked. Blocking of the second SH-group apparently did not cause any change in the ESR spectrum of the sample frozen in glycerol. This seems to suggest that the unpaired electron of the first label is located far from the second. However, the fingerprint analysis of the labelled and

digested protein has shown that the thiol group (Cys-153) near the reactive SH-group is modified. Since the length of spin-labelled reagent is about 8 Å, that of a peptide bond 3.5 Å, the unpaired electrons can be located from each other at a maximal distance of 27–33 Å. We assume that the two radicals are oriented in opposite directions, therefore no interaction can be demonstrated. From this it follows then that the peptide chain between Cys-149 and Cys-153 cannot be in helical conformation, since in case of a helix the maximal distance between the two radicals could not exceed 21 Å.

One of the authors (G. E.) is greatly indebted to Dr E. G. Rozantzev, in whose laboratory he studied the method of spin labelling. Thanks are due to Drs V. I. Suskina and A. B. Shapiro for kindly supplying the spin-labelled reagents and to Dr. A. I. Kokorin for letting us know his unpublished method. The authors also wish to thank Drs F. Tüdős and B. Mohos, as well as Mrs M. Szegvári, for their help throughout this work.

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The Regulatory Center of D-glyceraldehyde-3-phosphate Dehydrogenase

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Dedicated to Professor A. E. Braunstein in commemoration of his 70th birthday

Glyceraldehyde-3-phosphate dehydrogenase containing bound NAD is unable to bind tightly ATP. The enzyme-ATP complex can bind firmly only two moles of NAD with concomitant weakening of the enzyme-ATP binding.

The enzyme containing bound ATP forms completely the enzyme-NAD charge-transfer complex. Accordingly, ATP is not bound at the NAD-binding site in the active center.

The binding of adenosine phosphates (ATP, ADP, AMP) suspends the inhibition of enzymatic activity by excess NAD or inorganic phosphate. Adenine and adenosine affect only the inhibition by excess NAD.

We suggest that apart from the active site each subunit of glyceraldehyde-3-phosphate dehydrogenase has a specific regulatory site. Binding of NAD, adenosine phosphates or inorganic phosphate to this site decreases enzymatic activity.

Introduction

Glyceraldehyde-3-phosphate dehydrogenase isolated from mammalian muscle is a tetrameric protein (Harris, Perham 1965, Harrington, Karr 1965). The subunits of the enzyme have been shown to be chemically identical (Harris, Perham, 1968), although some data indicate the asymmetric association of subunits (Batke 1968, Malhotra, Bernhard 1968, Ovádi, Keleti 1969, MacQuarrie, Bernhard 1970).

According to recent data (Ovádi et al. 1971) two moles of ATP can be firmly bound instantaneously to the tetrameric apoenzyme without loss of enzyme activity. The binding of ATP induces the irreversible dissociation of the enzyme into dimers and the enzyme is inactivated. The kinetics of the inactivation suggest a complex mechanism of the simultaneous dissociation of tetramer and inactivation of both tetrameric and dimeric forms. The dissociation and inactivation is followed by the firm binding of two additional moles of ATP per mole of protein.

It has been demonstrated that excess NAD or inorganic phosphate inhibit the enzymatic activity. It was postulated that NAD at high concentration can be bound to a regulatory NAD-binding site (Batke, Keleti 1968).

Regulation may have statistical and biological significance (Weber 1963). In this paper we attribute the statistical significance to the term "regulation", since no data are available at present whether the inhibition by excess of NAD or P_i

(inorganic phosphate ion) has any physiological role in the *in vivo* regulation of glyceraldehyde-3-phosphate dehydrogenase activity.

In order to test the existence of the regulatory center, in this paper we have studied how ATP and related nucleotides influence the binding of NAD to the enzyme as well as the inhibition by excess substrate. Part of this work was presented at the Symposium of Friedrichroda (Ovádi 1972).

Materials and methods

Four times recrystallized swine muscle glyceraldehyde-3-phosphate dehydrogenase (Előd i, Szörényi 1956) was used.

D-glyceraldehyde-3-phosphate was prepared from fructose-1,6-diphosphate (Reanal) according to Szewczuk et al. (1961), ATP, ADP, AMP, adenosine, adenine and NAD (85% purity) were Reanal products. The other chemicals were commercial preparations of reagent grade.

Enzymatic activity with glyceraldehyde-3-phosphate and phosphate as substrates and the amount of firmly bound NAD of the enzyme were determined by the optical test as previously described (Keleti, Batke 1965, Batke 1968). The specific activity of the preparations varied between 15 000 and 20 000 NADH produced $\times$ min^{-1} $\times$ mole enzyme^{-1}. The molecular weight of the tetramer was taken as 145 000 (Előd i 1958). For removal of firmly bound NAD the enzyme was treated with charcoal, which resulted in an increase of the absorbance ratio (280 : 260 nm) from 1.05 to 1.95. Charcoal-treated enzyme (apoenzyme) was used in all experiments.

Protein concentration was determined on the basis of light absorbance at 280 nm, by using the extinction coefficients $A_{1\,cm}^{0.1\%} = 0.9$ and 1.0 for the apoenzyme and enzyme–NAD_4 complex, respectively. Protein samples which were not gel-filtered contained about 0.06 M SO_4^{2-} as determined with a Radelkisz conductivity meter.

The amount of tightly bound nucleotides could be determined after gel-filtration on a Sephadex G-50 column (1.5 $\times$ 20 cm) from the ratio of absorbancies at 280 and 260 nm, with the aid of an appropriate calibration curve, since we found that in these complexes the absorbancies of the enzyme and that of ATP and NAD were additive at both wavelengths. The molar extinction coefficients of ATP, NAD and NAD-free enzyme are 15 400, 19 800 and 66 000 at 260 nm and 2300, 3250 and 132 000 at 280 nm, respectively.

The enzyme contains 4 moles of bound NAD per mole of protein. Due to their different dissociation constants – of 10^{-11}, 10^{-9}, 10^{-7} and 10^{-5} order of magnitude, respectively (cf. Conway, Koshland 1968) – about 3 moles of NAD remain bound after gel-filtration of the protein.

The term "firmly bound" will be used to denote the nucleotides which remain in the protein fraction after gel-filtration. Gel-filtration was carried out at 0° in 0.1 M tris-HCl buffer, pH 8.5; the whole procedure lasted for about 15 minutes.

Treatment with ATP was carried out by adding to a 1×10^{-5} M protein solution in 0.1M tris-HCl buffer, pH 8.5, an ATP solution adjusted to the same pH. The mixture was kept at 0 °C in an ice water bath. Enzyme treated in a similar manner in the absence of ATP served as control. Treatment with the other nucleotides and adenine or adenosine was carried out in a similar mannor.

Opton PMQ II spectrophotometer and spectrophotofluorimeter was used with silica cells of 1 or 4 cm light path.

Results and discussion

Interaction between the binding sites of ATP and NAD

It is known that NAD bound to the active site of glyceraldehyde-3-phosphate dehydrogenase has a characteristic spectrum of the enzyme–NAD charge-transfer complex (Racker band). In order to decide whether ATP is bound by the NAD-binding site in the active center or not, we examined the effect of 4 moles of ATP per mole of protein on the formation of the "Racker band" and on the quenching of the protein fluorescence by NAD. The apoenzyme and the ATP-treated samples

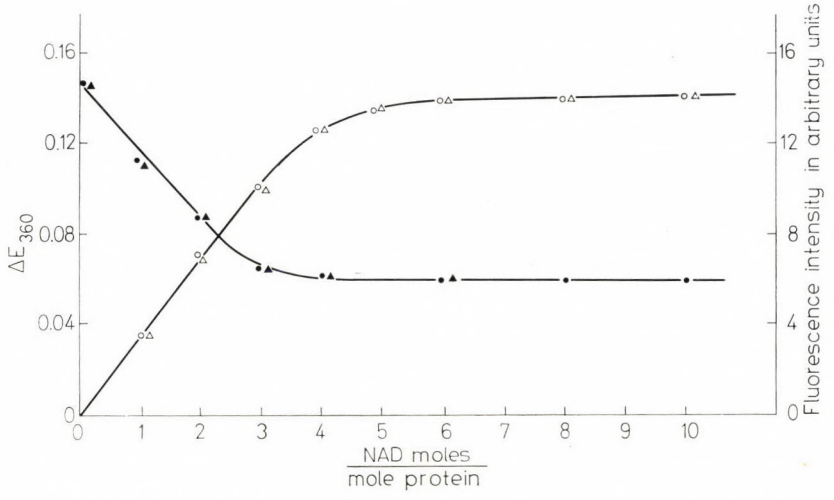

Fig. 1. The formation of Racker band and fluorescence quenching of ATP-treated apoenzyme by adding NAD. ATP treatment consisted in incubation of the apoenzyme with 4 moles of ATP per mole of protein for 10 minutes. The Racker band was measured at 1.4 mg/ml protein concentration in cells of 4 cm light path (left ordinate). ○ and △ represent the increase in absorbance on addition of NAD to apoenzyme and ATP-treated apoenzyme, respectively. The NAD-free enzyme had an absorbance of 0.052 at 360 nm, the molar extinction coefficient of the Racker band is 3400. Fluorescence was measured with protein solutions of 1.4 mg/ml in an Opton PMQ II spectrophotofluorimeter with silica cells of 1 cm light path, exciting at 380 nm and measuring the fluorescence at 460 nm. The fluorescence of NAD and ATP in the concentrations used was about 15 per cent of that of the protein solution. ● and ▲, change in fluorescence intensity on addition of NAD to apoenzyme and ATP-treated apoenzyme, respectively

were titrated with NAD. The extinction difference was measured at 360 nm whereas for the determination of fluorescence quenching the excimer fluorescence of the protein (Keleti 1970) was used. This phenomenon is suitable for the demonstration of enzyme–ligand complexes at high protein concentration (Keleti 1968) (Fig. 1).

We observed the same maximal change and the same NAD concentration dependence of the absorbance at 360 nm and of the fluorescence quenching with the ATP-treated and the untreated samples. It follows that ATP does not influence the formation of the NAD–enzyme charge-transfer complex, which takes place in the active center even after 3 hours of incubation with ATP when the enzyme has already lost 40 per cent of its activity.

Since the formation of the charge-transfer complex does not mean necessarily a tight binding of the coenzyme, we studied, (i) whether ATP-binding influences the firm binding of coenzyme and, (ii) whether NAD-binding interferes with the binding of ATP. In the first type of experiments we used apoenzyme incubated for 10 minutes with ATP in an about 2 to 4-fold molar excess, and added 4 moles of NAD. Alternatively, in the second type of experiments we first added NAD to the enzyme and then incubated it with ATP. Nucleotide binding was

Table 1

Interaction between the binding sites of ATP and NAD

Apoenzyme (1.4 mg/ml) was incubated with the nucleotides in 0.1 M tris-HCl buffer, pH 8.5, for 10 minutes. The second nucleotide, where indicated, was added separately, followed by another 10 minutes of incubation. The samples (2 ml) were gel-filtered on a Sephadex G-50 column (1.5 × 20 cm) equilibrated with 0.1 M tris-HCl buffer, pH 8.5; 2.5 ml fractions were collected. NAD content was measured enzymatically in the following mixture: 0.1 M glycine buffer, pH 8.5, 1×10^{-3} M disodium arsenate, 2.5×10^{-3} M glyceraldehyde-3-phosphate, using about 300 μg/ml enzyme. The nucleotides are indicated in the order of addition. Nucleotide content was determined from the ratio of absorbancies at 280 and 260 nm. This determination had an error of $\pm 10\%$

Samples	Nucleotides measured before gel-filtration moles per mole protein	Nucleotides in the protein peak	
		NAD moles per mole protein	ATP moles per mole protein
Holoenzyme	3.6	2.7	—
Apoenzyme + 4NAD	4.1	2.8	—
Apoenzyme + 2ATP	2.1	—	1.8
Apoenzyme + 4ATP	4.2	—	1.8
Apoenzyme + 2ATP + 2NAD	4.3	2.0	0.8
Apoenzyme + 4ATP + 4NAD	7.9	2.0	0.9
Apoenzyme + 4NAD + 4ATP	8.2	2.8	0.0

tested by gel-filtration as described in Methods. The amount of bound nucleotides was determined from the absorbance ratio (280 : 260 nm) of the protein peak, whereas the NAD content of the protein peak was determined enzymatically (Table 1).

As shown in Table 1, about 3 moles of NAD and about 2 moles of ATP per mole of protein are recovered in the gel-filtered enzyme–NAD and enzyme–ATP complex, respectively. If NAD was added to the apoenzyme–ATP complex only 2 moles of NAD and 1 mole of ATP per mole of protein were present in the gel-filtered samples. On the other hand, if NAD was first added to the apoenzyme, this prevented any firm binding of ATP. These results suggest that the firm bindings of ATP and NAD mutually influence each other and perhaps one may assume that exclude each other in the same subunit.

The effect of ATP and its analogues on the inhibition by excess substrates

According to earlier data excess NAD causes maximally an about 40% inhibition of enzymatic activity (Batke, Keleti 1968). Since we have shown that ATP is not bound to the binding site of NAD in the active center, and ATP can be regarded as a structural analogue of the ADP moiety of NAD, we examined the effect of 2 and 4 moles of ATP per mole of protein on the inhibition by excess NAD (Fig. 2).

Fig. 2A shows the results obtained with the apoenzyme–$(ATP)_2$ complex. Immediately after addition of 2 moles of ATP per mole of protein, the enzyme is practically fully active. Moreover, a partial protection against inhibition by excess NAD is observed.

If the apoenzyme is incubated with 4 moles of ATP per mole of protein, partial protection against inhibition of excess NAD occurs instantaneously and, as inactivation proceeds full protection has been found (Fig. 2B). The irreversible inactivation may be related to the secondary oxidation of SH-groups which became unmasked during the dissociation of the enzyme into dimers (Ovádi, Keleti 1972). If 50 moles of NAD per mole of protein are added to the enzyme previously incubated for about 1 hour with 4 moles of ATP per mole of protein, inactivation is slowed down and the active molecules exhibit the same anomalous saturation curve as those treated with 2 moles of ATP per mole of protein (cf. Fig. 2A).

We have also examined the immediate effect of up to 1000-fold molar excess of ATP on the inhibition by excess NAD. We did not find higher protection than in the presence of 2 or 4 moles of ATP.

These findings support there assumption that there exists a specific binding site (a regulatory center) to which adenosine phosphates can be liganded. Furthermore in the apoenzyme only 2 subunits can bind ATP and two further equivalents of ATP can be bound to the regulatory centers only after some changes in the protein. Previous experiments (Ovádi et al. 1971) indicate that these changes may be connected with the dissociation of the tetrameric enzyme into dimers.

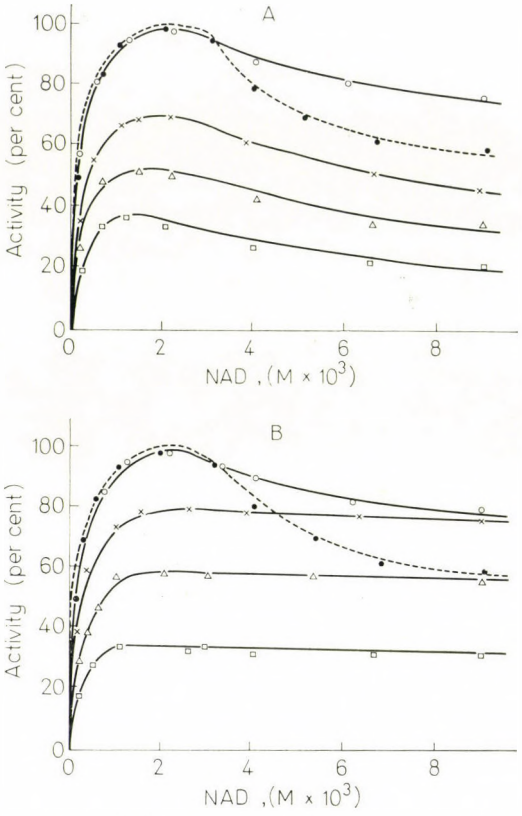

Fig. 2. Effect of ATP on the inhibition by excess NAD. Apoenzyme (1.4 mg/ml) was incubated with ATP in 0.1 M tris-HCl buffer, pH 8.5, at 0 °C. Enzymatic activity was measured with 1 μg/ml enzyme in the following mixture: 0.1 M glycine buffer, pH 8.5, 1×10^{-2} M disodium hydrogen phosphate, 2.5×10^{-3} M D-glyceraldehyde-3-phosphate and different concentrations of NAD. A: apoenzyme incubated with 2 moles of ATP per mole of protein. ● : untreated enzyme; ○, ×, △ and □: enzyme incubated with ATP for 2, 60, 180 and 240 minute,s respectively. B: Apoenzyme incubated with 4 moles of ATP per mole of protein. ●: untreated enzyme; ○, ×, △ and □: enzyme incubated with ATP for 2, 60, 150 and 240 minutes, respectively

Fig. 3 shows that excess P_i causes the decrease of enzymatic activity up to 70% residual activity (cf. Keleti 1972). The effect of 4 moles of ATP per mole of protein on the inhibition by excess P_i is also presented in Fig. 3. We found that the anomalous saturation curve becomes an approximately hyperbolic one after prolonged incubation with ATP. From these results it seems probable that the phosphate group of ATP is bound to a specific P_i binding site. We have excluded the possibility that this binding site is the same as that of the phosphate moiety of NAD, since we have found that excess NAD does not suspend the inhibitory action of excess P_i and *vice versa*.

We studied the effect of ADP, AMP, adenosine and adenine on the inhibition caused by excess NAD or P_i, in order to examine the role of phosphate and ribose moiety in the binding of nucleotides and to obtain information about the distance between the two binding sites in the regulatory center.

On addition of 4 moles of ADP or AMP per mole of protein, similarly to ATP about 2 moles of ADP and AMP per mole of protein are bound firmly and instantaneously to the enzyme. Examining the suspension of the inhibition caused by excess NAD or P_i, we obtained practically the same patterns as in Figs 2B and 3.

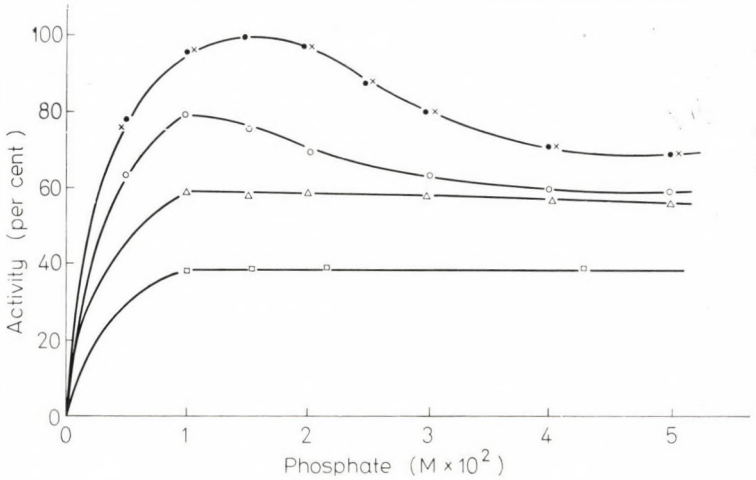

Fig. 3. Effect of ATP on the inhibition by excess phosphate. Conditions as in Fig. 2, NAD concentration was fixed (2×10^{-3} M) and that of phosphate was varied. ● : untreated enzyme; ×, ○, △ and □: enzyme incubated with 4 moles of ATP per mole of protein for 2, 60, 180 and 240 minutes, respectively

In contrast to the above results on addition of 4 moles of adenine or adenosine per mole of protein no rapid and firm binding has been found and these substances did not protect instantaneously against inhibition by excess NAD, only after about 3 hours of incubation. Inhibition by excess phosphate was not influenced by adenine or adenosine even after prolonged incubation. Figs 4 and 5 show the results obtained with adenine treatment.

The results suggest that the phosphate group of adenosine phosphates plays an important role in the orientation and binding of these nucleotides to the protein.

As adenine and adenosine can also be bound to the enzyme on prolonged incubation, and they are able to suspend the inhibition by excess NAD, it appears reasonable to assume that the adenine ring of the nucleotides interacts with the regulatory NAD-binding site. Since binding of the nucleotides protects the enzyme against inhibition by excess P_i, but binding of adenine or adenosine has no such effect, we propose that ATP, ADP and AMP can be bound at two points in the

regulatory center of the enzyme: through the adenine ring and the phosphate group. If this is true, the distance between the binding sites of the adenine moiety and phosphate in the regulatory center seems to be not greater than the length of AMP.

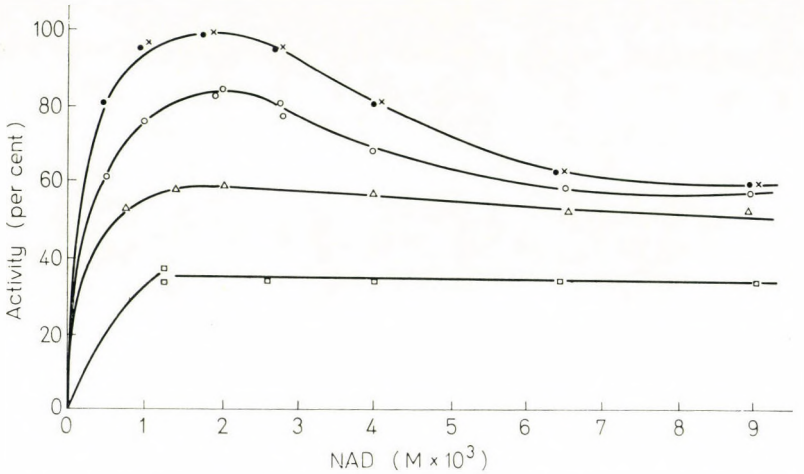

Fig. 4. Effect of adenine on the inhibition by excess NAD. Conditions as in Fig. 2 but the enzyme was incubated with 4 moles of adenine per mole of protein. ●: untreated enzyme; ×, ○, △ and □: enzyme incubated with adenine for 2, 120, 180 and 330 minutes, respectively

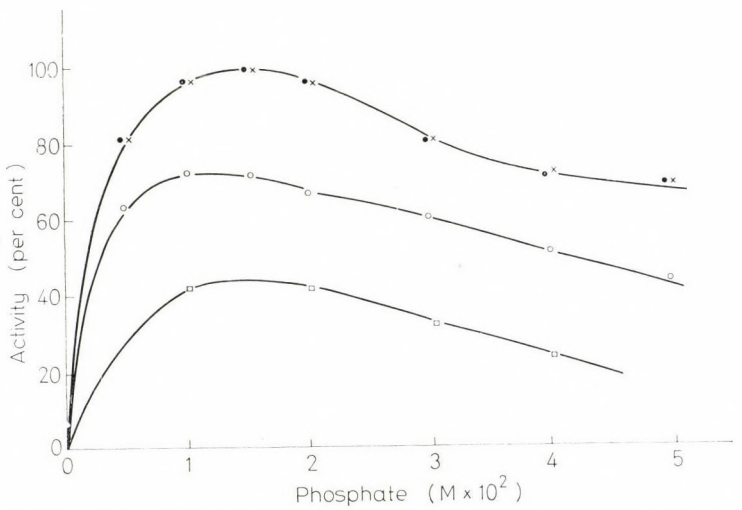

Fig. 5. Effect of adenine on the inhibition by excess phosphate. Conditions as in Fig. 3, but the enzyme was incubated with 4 moles of adenine per mole of protein. ●: untreated enzyme: ×, ○ and □: enzyme incubated with adenine for 2, 180 and 330 minutes, respectively

The binding of adenosine phosphates may be interpreted in two alternative ways: *a)* The phosphate group of nucleotides may be captured by some region of the apoenzyme *via* electrostatic interaction and the nucleotides are bound to the adenine binding site of the regulatory center. After the binding of the adenine moiety the phosphate groups of the nucleotides are rearranged to the P_i binding site of the regulatory center. This implies a competition between the excess of P_i and the phosphate groups of adenine nucleotides. *b)* The adenine and phosphate moieties of adenosine phosphates are simultaneously bound to the regulatory center and the liganding of phosphate moiety induces a steric change by which the other P_i binding site(s) become(s) buried. In any way the binding of the phosphate moiety of adenine phosphates accounts for the suspension of inhibition by excess P_i.

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A New Enzymatic Method for the Determination of Acetoacetate

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An enzymatic method for the quantitative determination of acetoacetate is described. The enzymes required for the method are comprised in a 40–70% acetone fraction prepared from pigeon liver acetone powder. These enzymes are acetoacetyl-CoA synthetase, β-ketoacyl thiolase and arylamine acetylase. The method is very simple and sensitive.

Introduction

Stern et al. (1949, 1951, 1953) have observed that citrate is formed from acetoacetate in the presence of ATP,* CoASH and oxaloacetate by pigeon liver extracts. When SA is used instead of oxaloacetate as acetyl acceptor, acetyl-SA is produced. Of the β-ketoacids of higher C atom number, for example, β-ketovaleric acid, β-ketocaproic acid and β-ketooctanoic acid may also serve as acetyl donor in the above order of decreasing reactivity.

Extracts prepared from kidney and yeast (Stern et al., 1953) can also utilize acetoacetate as acetyl donor. This transformation involves first the activation of acetoacetate, then the cleavage of the β-ketoacyl-CoA ester produced and finally the transfer of acetyl group from CoA to SA or oxaloacetate.

The above facts render it possible, in principle, to determine acetoacetate by the aid of pigeon liver extracts. We observed that on the effect of a 40–70% acetone fraction prepared from pigeon liver acetone powder 2 moles of acetyl-PNA were produced from 1 mole of acetoacetate in the presence of ATP, CoASH and PNA. It was this observation which formed the basis of the elaboration of a method for the quantitative determination of acetoacetate.

Methods

Acetoacetate was prepared from freshly distilled acetoacetic ester according to Ljunggren (1924), and ketoacid content was determined enzymatically by the aid of D-(-)-β-hydroxybutyrate dehydrogenase (Mellanby, Williamson, 1962).

* *Abbreviations:* ATP, adenosine 5'-triphosphate; CoASH, coenzyme A; SA, sulfanylamide; PNA, p-nitroaniline; EDTA, ethylene diamine tetraacetate.

Pigeon liver acetone powder was prepared according to Kaplan and Lipman (1948) and was fractionated as follows. Acetone powder (2.4 g) was extracted with 24 ml of cold (0–3 °C) distilled water for 30 min at 0 °C with continuous stirring and the insoluble residue was removed by centrifugation (12 000 r.p.m.). The supernatant (19.2 ml) was mixed with acetone (15.2 ml) cooled to −15 °C to give a final acetone concentration of 40%. Acetone was added over a period of 10 min. The protein precipitate was removed by centrifugation for 15 min at 12 000 r.p.m. To the supernatant 38.6 ml acetone cooled to −15 °C was added to give 70% in acetone. The protein precipitate was sedimented by centrifugation at 12 000 r.p.m. and dissolved in 3 ml of 0 °C distilled water. The solution was frozen overnight, then thawed and again centrifuged. The supernatant thus obtained served as our active enzyme preparation, which was used without further treatment. The protein concentration of the preparation is about 12 mg/ml as determined by the method of Ditterbrandt (1948). The enzyme preparation is stable at 0–5 °C for at least a week, and in the freeze-dried state it can be stored for at least four months without any detectable loss in activity.

Activity was assayed by the continuous PNA-method of Tabor et al. (1953), as described for the determination of acetyl-S-CoA (Decker, 1962). The composition of the reaction mixture is shown in Table 1. Spectrophotometric readings

Table 1

Composition of reaction mixture

Reagents	Control mixture, ml	Test mixture. ml
Phosphate buffer, 0.2 M, pH 6.8 (according to Sörensen)	1.0	1.0
Mercaptoethanol, 0.1 M	0.1	0.1
EDTA, 0.1 M	0.1	0.1
KOH, 0.5 N	0.02	0.02
ATP, 0.1 M	0.1	0.1
$MgCl_2$, 0.1 M	0.1	0.1
CoASH, 0.0013 M	0.05	0.05
PNA, 0.002 M	0.05	0.1
Sample	—	up to 0.5
Distilled water	up to 2.22	2.22

are taken at 405 nm. After the addition of 0.1 ml enzyme solution and proper mixing the initial absorbancy value (A_1) is read and the decrease in absorbancy is followed until it reaches the final value (A_2). Reaction volume: 2.32 ml, light path: 1.0 cm. The measurement is carried out at room temperature against an appropriate control. The calculation is given by the formula

$$\frac{(A_1 - A_2) \times \text{ml test solution}}{\Sigma \times 2} = \mu\text{moles of acetoacetate in the test solution}$$

where A_1 = initial absorbancy value at 405 nm,
A_2 = final absorbancy value at 405 nm,
Σ = 10.25 $cm^2 \times \mu mole^{-1}$, the μmolar extinction coefficient of PNA at 405 nm (Decker, 1962).

In order to test our method on a biological material we applied human blood, the acetoacetate content of which was also determined by the method of Mellanby and Williamson (1962).

Results and discussion

The method for acetoacetate determination depends on ATP and CoASH concentrations as shown in Fig. 1. In the absence of ATP there is no reaction, whereas if CoASH is omitted a slow process is observed, which might be attributed to the endogenous CoASH content present in the enzyme preparation. The enzyme preparation contains β-ketoacyl thiolase activity (Decker, 1962). If PNA is in excess in the reaction mixture, then acetyl-PNA is produced quantitatively on the effect of arylamine acetylase, i.e. acetoacetate is completely converted into acetyl-PNA.

Table 2 illustrates the reliability of the method. The upper limit for acetoacetate is 50 nmoles per tube, the lower limit is about 5 nmoles.

As shown by our experiments the enzyme preparation is highly specific for the determination of acetoacetate. The CoA esters of higher β-ketoacids are split by thiolase, but these higher β-ketoacids are probably not activated in the above system. Thus e.g. triacetic acid does not react at all. All of the following substances gave negative results when tested as possible acetyl donors: triacetic acid lactone, citrate, acetate, β-methyl crotonate and acetone.

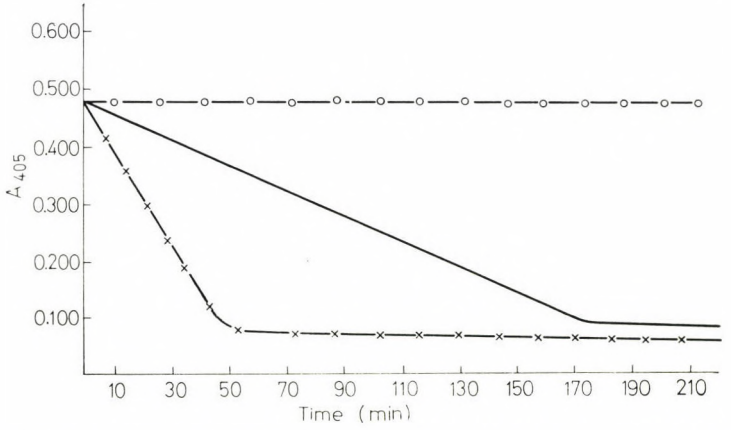

Fig. 1. Effect of ATP and CoASH on the progress of enzyme reaction. The composition of reaction mixture cf. in Methods and Table 1. × — ×, whole system; ○ — ○, whole system without ATP; ————, whole system without CoA

Table 2

Recovery of authentic acetoacetate added to the mixtures by the continuous PNA method

The composition of reaction mixture cf. in Table 1. The experiments were carried out and calculated as described in Methods. Acetoacetate solutions were calibrated by means of the β-hydroxybutyric acid dehydrogenase method

No.	Acetoacetate		
	Added, nmoles	Measured, nmoles	Recovery, per cent
1	40.0	41.0	102.0
2	40.0	42.0	105.0
3	42.0	41.0	97.6
4	20.5	20.0	97.5
5	4.5	4.5	100.0

Table 3

Determination of the acetoacetate content of human serum before and after the addition of authentic amounts of acetoacetate

Acetoacetate solutions were calibrated by the β-hydroxybutyrate dehydrogenase method of Mellanby and Williamson (1962). As to the continuous PNA-method cf. Table 1. To the serum acetoacetate was added to give an 0.2 mg/100 ml increase in concentration. The recovery of added acetoacetate is expressed in per cent

No.	β-hydroxybutyrate dehydrogenase method			Continuous PNA-method		
	Before addition, mg/100 ml	After addition, mg/100 ml	Recovery, per cent	Before addition, mg/100 ml	After addition, mg/100 ml	Recovery, per cent
1	0.40	0.59	95	0.41	0.60	95
2	0.41	0.60	95	0.42	0.60	90
3	0.40	0.59	95	0.42	0.61	95

Table 3 presents the results of our determination of the acetoacetate content of human sera, as compared with the data obtained with the procedure utilizing β-hydroxybutyrate dehydrogenase. In addition, the recovery of authentic acetoacetate added to the biological material is also indicated.

In conclusion, the above method seems to be suitable for both clinical and biochemical purposes. Its advantages can be summarized as follows:

1. Our method is about 2–3 times as sensitive as the procedure based on β-hydroxybutyrate dehydrogenase, which has so far been the most accurate method described in the literature.

2. The preparation of our enzyme extract is much simpler than that of β-hydroxybutyrate dehydrogenase from Rhodopseudomonas spheroides (Mellanby, Williamson, 1962).

3. Measurements at 405 nm are feasible on simple photometers.

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Demonstration of an Enzymatic Transfer Reaction between β-Methyl-crotonyl-Coenzyme A and L-Carnitine

(Short Communication)

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During the last ten years a considerable body of evidence has been accumulated for the important role of the macroergic compounds of various acids with L-carnitine in the living cell, in addition to the CoA* esters (Bressler, 1970). The reversible transfer of various acyl groups from the SH-group of CoA to the OH-group of L-carnitine is brought about by specific transferases. These partly mitochondrial and partly extramitochondrial enzymes are presumably involved in transport and regulatory processes (Bressler, Brendel, 1966). First the acetyltransferase enzyme (Acetyl-CoA: carnitine O-acetyl-transferase, EC 2.3.1.7) was discovered by Friedman and Fraenkel (1955) in an extract of pigeon liver acetone powder. Fritz et al. (1963) succeeded in the purification of this enzyme from pig heart to a specific activity of 4.6. Fritz et al. reported data about the specificity with regard to carbon atom number, but these results are not very reliable because the enzyme preparation used was not sufficiently pure. However, it is noteworthy that this enzyme was completely inactive towards palmityl-CoA. The enzyme was crystallized by Chase et al. (1965) from pigeon breast muscle and this preparation had a specific activity of 119. However, these authors did not examine the specificity with regard to carbon atom number. The enzyme catalyzing the transfer of palmitoyl group was demonstrated by Bremer (1962) in rat heart mitochondrial membrane (Norum, Bremer, 1967). Recently Solberg (1971) found in the carnitine-acetyl-transferase preparation (specific activity: 80) of Boehringer Ltd. a foreign activity which differed from the acetyltransferase enzyme and was specific for fatty acids containing 6 to 10 carbon atoms. Solberg denoted this enzyme as carnitine-octanoyl transferase. The investigations described so far are pertinent only to the carnitine esters of aliphatic fatty acids.

In the present work we examined whether the following reaction can be catalyzed enzymatically:

$$\beta\text{-methyl-crotonyl-CoA} + \text{L-carnitine} \rightleftarrows \beta\text{-methyl-crotonyl-L-carnitine} + \text{CoA}$$

The search for such an activity is justified by the fact that β-methyl-crotonyl-CoA plays an important role in steroid synthesis (Decker, 1959).

In the experiments we used carnitine-acetyltransferase (Boehringer) of specific activity 80. The β-methyl-crotonyl-CoA was prepared according to the

* *Abbreviations:* CoA, coenzyme A; EDTA, ethylenediamine tetraacetic acid.

method of Knappe et al. (1961), acetyl-CoA was prepared as described by Stadtman (Colowick, Kaplan, 1957). The above reaction was measured from left to right and the amount of CoA liberated was determined with Ellman's reagent. As shown in Table 1, the hypothetic enzyme reaction indeed takes place, and at a reasonable rate. As suggested by Fritz et al., the rate of the transfer reaction was

Table 1

Comparison of the enzymatic transfer reactions of acetyl-CoA and β-methyl-crotonyl-CoA

The cuvettes contained the following components in 2.3 ml final volume: 200 μmoles of Tris-HCl, pH 7.8; 10 μmoles of EDTA; 200 nmoles of 5,5'-dithiobis (2-nitrobenzoic acid); 200 nmoles of DL-carnitine; 56 nmoles of acyl-CoA; carnitine-acetyl transferase as indicated. The reaction was started by the addition of enzyme and the mixture was then incubated at 25 °C for 30 min. At the end of the incubation period the change in absorbance at 412 nm was read and the calculations were based on the molar extinction coefficient: 13 600 M^{-1} cm^{-1}. The reference cuvette did not contain carnitine

Substrate	CoA liberated in the first 30 min, nmoles		
	0.009 enzyme units	0.018 enzyme units	0.04 enzyme units
Acetyl-CoA	19.2	26.4	39.0
β-methyl-crotonyl-CoA	9.6	15.0	18.4

compared with that of the transfer reaction of acetyl-CoA measured within the same period. As for the nature of the enzyme catalyzing the transfer reaction of β-methyl-crotonyl group, at present we are only confined to speculations:

1. The broad specificity of the acetyl-transferase enzyme may also include the substrate tested in this work, but the reaction proceeds at a low speed.

2. The reaction observed by us may be attributed to the octanoyl-transferase activity described by Solberg (1971).

3. Finally, we may deal with an entirely new enzyme.

In order to validate one of the above alternatives further experiments are required.

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Isolation of Nuclear Ribonucleoprotein Particles Containing D-RNA from Rat Liver by Gel-filtration

(Short Communication)

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The investigation of the proteins of the D-RNA containing ribonucleoprotein component of the cell nuclei requires the production of a great quantity of the particles. Usually the nuclear extract containing 30 *S* particles is centrifuged in a sucrose gradient (Samarina et al., 1967) and the material containing the 30 *S* particles is collected. Sufficiently pure particles may be obtained in this manner, but the method is rather time consuming and requires large bucket- or zonal-rotors.

In this paper a method will be described by which a large quantity of particles can be rapidly purified by means of gel-filtration.

Two rats (R Amsterdam) weighing 150 g were used for each batch. The animals were administered 20 μCi ^{14}C-orotic acid intraperitoneally 30 minutes before death. Cell nuclei were prepared by the modified Chauveau method and 2 ml of nuclear extract were obtained (Samarina et al., 1967). The extract was gel-filtered through a Sephadex G–200 column equilibrated with 140 ml of STM buffer (0.1 M NaCl–0.01 M triethanolamine-HCl–0.001 M $MgCl_2$ (pH 7.5). The length to diameter ratio of the column was 20 : 1, flow rate 14 ml/hour. After 40 ml buffer passed through the column, fractions of 10 drops were collected by an automatic collector.

The ultraviolet absorption at 260 nm was measured from 50 μl samples of the fractions. In addition, the radioactivity was measured by adding an adequate quantity of ribosomal RNA as a carrier to the original preparation and by washing the acid-insoluble material of the fractions with 5% trichloroacetic acid on to a millipore filter (Synpor 2). Radioactivity was determined with a GAMMA counter in an apolar scintillator liquid (100 mg POPOP, 4 g PPO in 1000 ml toluene) (Fig. 1). Fractions 22 to 33 were pooled and the properties of the particles were examined as follows.

The filtrate (0.4 ml) was layered over a 15–30% sucrose gradient and centrifuged in the 3 × 5 ml rotor of a Janetzki VAC 60 ultracentrifuge at 38 000 r.p.m. at 4 °C. Fractions of 3 drops were collected (Fig. 2b). It can be seen that gel filtration separates the 30 *S* particles well from the small molecular weight impurities of the nuclear extract (sedimenting in the 4–6 *S* zone) (Fig. 2a).

A part of the material of the chromatogram was fixed in 2% neutralized formaldehyde and then dialyzed against a 0.005 M phosphate buffer (pH 7.4)

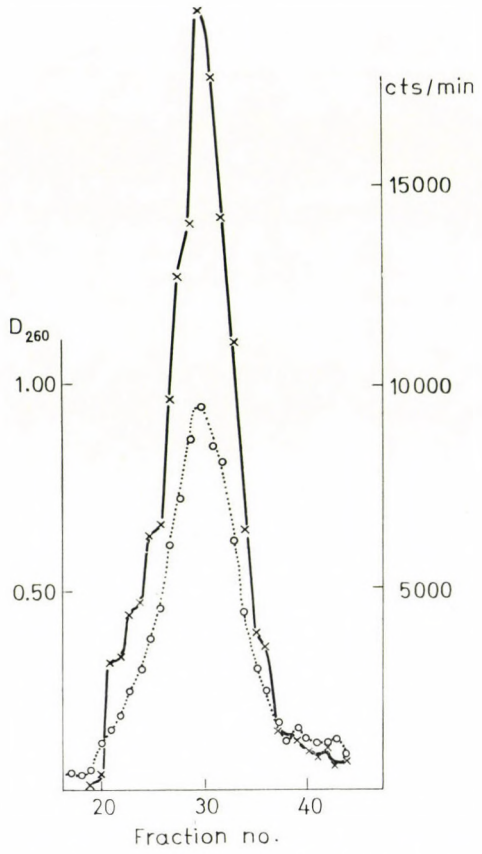

Fig. 1. Elution profile of nuclear extract on Sephadex G-200 column (The conditions of the chromatography are described in the text). ○ · · · ○, absorbancy at 260 nm; × — ×, radioactivity

containing 2% formaldehyde. The dialyzate was mixed into a preformed CsCl gradient and centrifuged until equilibrium was reached (Samarina et al., 1967a). Fractions of 3 drops were collected and their ultraviolet absorption at 260 nm and radioactivity were measured (Fig. 3). The density of every fifth fraction of the gradient was estimated in a refractometer. The density of the particles ($\rho = 1.405$ gcm^{-3}) purified by gel-filtration agreed well with that of the 30 *S* particles produced by the sucrose gradient method (Samarina et al., 1967a). A single homogeneous fraction could be found, where the ultraviolet absorption peak coincided with the radioactivity peak.

Another portion of the chromatogram was used for the electronmicroscopical examinations. These samples were fixed in 2% neutralized formaldehyde. The fixed material was dialyzed through a 2% parlodium membrane against a STM buffer, pH 7.2 (the STM contained 0.01 M NaCl). Single drops of the dialyzed

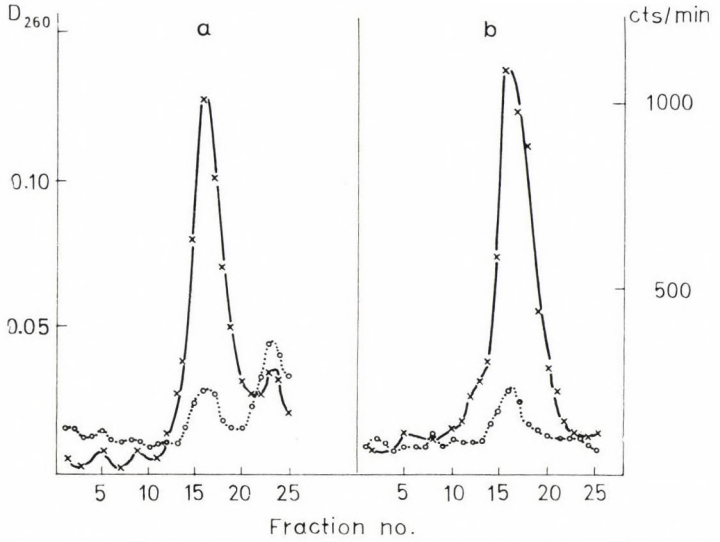

Fig. 2. Sedimentation pattern of the nuclear extract (a) and the 30 *S* particles purified by gel-filtration (b). a) 0.3 ml of the nuclear extract was layered over 4.8 ml of a 15—30% sucrose gradient. b) 0.4 ml of a preparation purified by gel-filtration was layered over the gradient. The gradient was prepared with a STM buffer, pH 7.5 (The conditions of the centrifugation are described in the text, the symbols are the same as in Fig. 1)

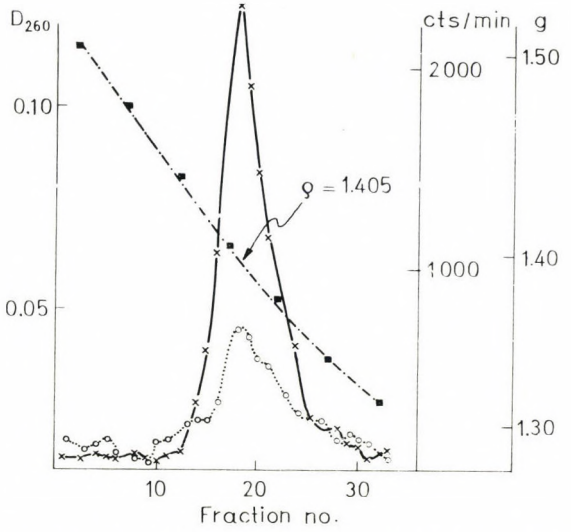

Fig. 3. Sedimentation pattern of 30 *S* particles (purified by gel-filtration and fixed in formaldehyde) in a preformed CsCl equilibrium density gradient ($\rho = 1.30-1.50\ gcm^{-3}$). Ultracentrifugation was carried out in the 3 × 5 ml rotor of a Janetzki VAC 60 ultracentrifuge for 16 hours at 36 000 r.p.m. at 10 °C. The symbols cf. Fig. 1

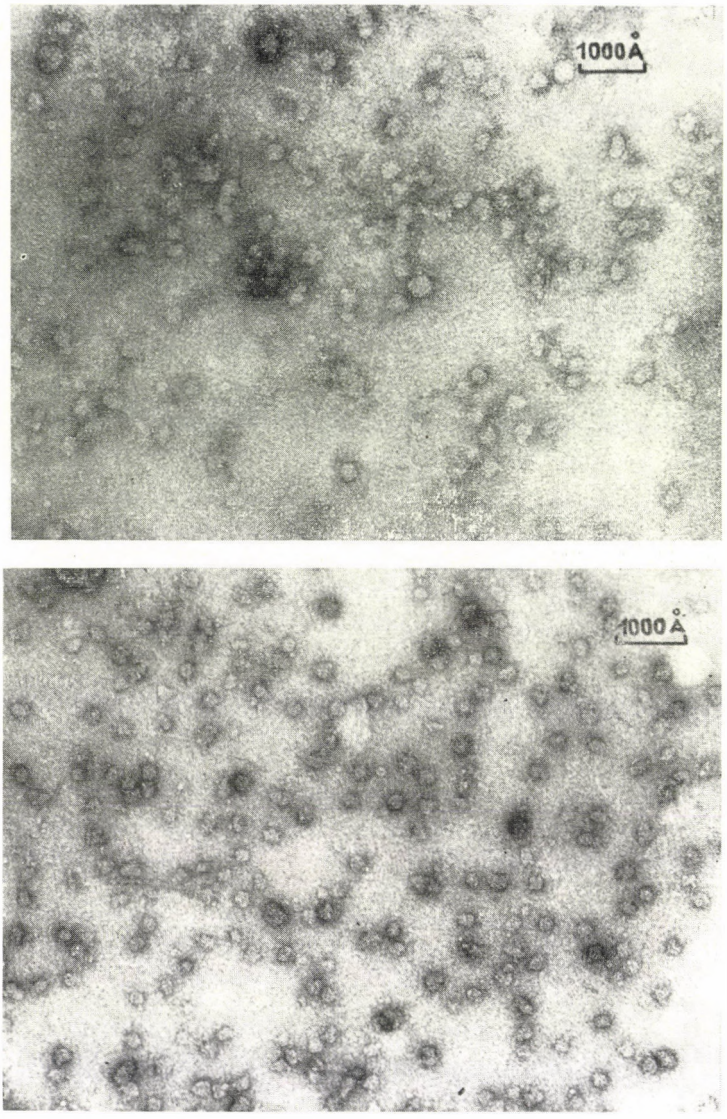

Fig. 4. Electronmicrographs of 30 *S* particles purified by gel-filtration (a) and obtained by the sucrose gradient method (b). Negative staining with uranyl acetate. × 115 000

material were placed on grids covered with parlodium membrane, and the preparations were stained with a 2% aqueous solution of uranyl acetate by the negative contrast technique. After removal of dye solution the preparations were examined under a Tesla BS 513 type electron microscope at 80 kV accelerating voltage with a 35 900-fold basic magnification. It could be seen in the electron

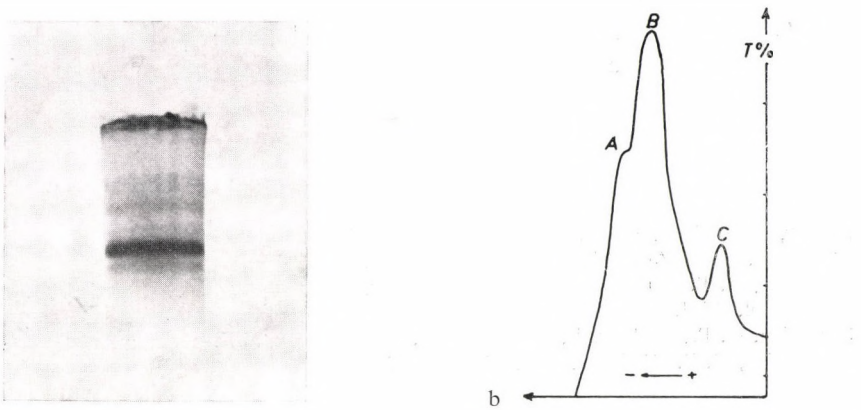

Fig. 5. Electropherogram of an informofer preparation purified by gel-filtration (a) and the direct densitogram of the gel (b). Disc electrophoresis in a cationic system containing 6 M urea. 150 μg protein, 5 mA/tube, 200 minutes. The inner diameter of the tube was 6 mm

micrographs that the chromatogram contained globular particles 180–200 Å in size (Fig. 4a). If these micrographs are compared to those of the 30 *S* particles obtained by the 15–30% sucrose gradient method (Fig. 4b), it seems that the particles yielded by the two procedures display practically identical submicroscopic characteristics.

The 30 *S* particles purified by chromatography were treated with RNase in the presence of 6 M urea. Then the proteins were washed with trichloroacetic acid and analyzed by polyacrylamide gel electrophoresis in a cationic discontinuous gel and buffer system containing 6 M urea (Molnár, 1969). The electropherograms were photographed (Fig. 5a) and direct densitometry was carried out with a Zeiss Schnellphotometer in an adapter cuvette (Fig. 5b) (Juhász, Molnár, 1972). Three major protein components could be found (A, B and C) whose electrophoretic mobilities and relative quantities agreed well with earlier data (Molnár, 1969).

The above experiments were repeated on a BioGel A 5 m column with the nuclear extract. Practically the same results were obtained.

It seems that by our procedure 30 *S* particles, containing informofer and D-RNA, can be prepared by gel-filtration through Sephadex G-200 column from the nuclear extract. No differences could be detected between the particles prepared by the sucrose gradient centrifugation and by our method.

The main advantages of our technique are as follows:

The nuclear ribonucleoprotein particles containing D-RNA can be isolated without gradient ultracentrifugation. The yield and particle concentration of the preparation is 2–3-fold that of the conventional method. Sucrose need not be removed from the preparation by dialysis, and the application of highly pure RNase-free sucrose is unnecessary.

The particles can be isolated in a very simple manner and in half the time usually needed. This results in a proportional decrease of the danger of degradation which may occur in the course of preparation.

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Forces Acting Between Muscle Filaments, I

Filament Lattice Spacing in Bee Flight Muscle

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Contrary to vertebrate striated muscle the myofibrils of the bee wing muscle do not behave as a body of constant volume. The decrease of filament spacing is much stronger than expected on the basis of the principle of volume constancy. The volume decrease of the myofibrils seems partly compensated by an enormous expansion of the mitochondria. We attribute the changes of the lateral separation to the balance of long-range forces the resultant of which may strongly depend on the degree of the overlap of the two kinds of myofilaments.

Introduction

The lateral changes of the muscle accompanying its length changes have been regarded as an important feature by different authors. According to Ernst (1963, 1968) theories dealing with shortening of muscle must also account for simultaneous widening. According to Belágyi (1969) and Ernst (1970) the widening of muscle can be described by a mathematical function similar to the time course of contraction.

A problem in close relation to lateral widening, i.e. the changes of the lateral distance of myofilaments, became recently a highly actual question of muscle research. Several authors (Huxley, 1952; Elliott et al., 1967) stated that by length changes the spacing of the filament lattice will change in such a manner that the volume of muscle remains constant. This means that the lattice constant of the crystalline lattice of filaments changes in a reverse proportion to the square root of sarcomere length.

On the basis of this statement it is now widely accepted that the filament distance is determined by the constancy of the volume of muscle (or of the muscle cell). Huxley (1960) wrote: "In the first place, as the volume of the muscle fiber remains constant during a stretch, the cross-sectional area must decrease and hence the side spacing between the filaments must decrease . . ."

Several concepts and theories of contraction based on the constant-volume behaviour of the muscle were published in the last few years (Elliott et al., 1970; Dragomir, 1970; Shear, 1970). It is a common feature of all of them that they regard the volume-constancy as the cause of contraction and not as a consequence of length changes. According to our opinion it is mainly the myofibrillar appa-

ratus which determines the volume of the muscle, since forces acting between myofilaments depending on the length (i. e. on the degree of overlap) may change in the manner that the changes filament distance do not cause any considerable change of volume in case of vertebrate muscle.

It will be shown that in the flight muscle of bee the filament spacing is not regulated by the constancy of volume and its length-dependence seems to differ from that of vertebrate muscle. This indicates the functional importance of the phsyical forces acting between the two kinds of filaments in the resting muscle.

In our opinion the solution of this latter question is particularly important from the point of view of the elasticity of resting muscle. It is now generally accepted that the physical forces acting between the two kinds of filaments do not contribute to the resting elasticity. According to Huxley (1969) the actin filaments may move freely alongside the myosin filaments on cushions of long-range forces. The elastic properties of the resting muscle are now generally attributed to parallel stuctures, sarcolemma, cell membrane and longitudinal sarcoplasmic reticulum. It is to be mentioned that nowadays even the term "muscle elasticity" seems to be disappeared from the literature (Ernst, 1970).

Materials and methods

The dorsoventral wing muscle of bees has been fixed at different lengths according to the method described in our earlier papers (Garamvölgyi, Belágyi, 1968; Garamvölgyi, 1969). The fixation was performed in osmium tetroxide (1 per cent) for one hour, immediately after the preparation of the specimens.

For embedding we used Durcupan-Fluka ACM araldite. The sectioning was carried out by an LKB "Ultrotome III" ultramicrotome. The sections were stained by uranyl acetate – lead citrate and studied in a WF SEM-3 electron microscope. The preparation of longitudinal and transverse sections from the same specimen was performed exclusively by means of the different specimen holders of the LKB ultramicrotome. Neither re-embedding nor any special device (e.g. Ernst et al., 1969) have been used.

Observations

Contrary to flight muscles of other insects (fly, Hanson, 1956; waterbug, White, 1966; Pringle, 1967) which tear by an insignificant elongation, the flight muscle of the bee can be stretched to its threefold length (Garamvölgyi, 1966; Garamvölgyi, Belágyi, 1968; Ernst et al., 1969). It is the first half of the total range of extensibility which fairly coincides with the range of the sliding movement (Belágyi, Garamvölgyi, 1968). This range corresponds to sarcomere lengths from 2.8 μ to about 5.4 μ, i.e. it extends from a virtually total overlap to a virtually non-existing overlap. The overlap, however, does not cease at its calculated end, re-

sidual overlap zones of 1000 – 1200 Å length persist and the further stretch happens by the own elongation of the myosin filaments (Garamvölgyi, 1969). Accompanying this increase of length the distance between myosin filaments decreases (Fig. 1a and b). This decrease seems considerable. The centre-to-centre distance in Fig. 1b representing a highly stretched fibril decreased to about half of the filament distance of the fibril fixed at its in situ length, as shown in Fig. 1a.

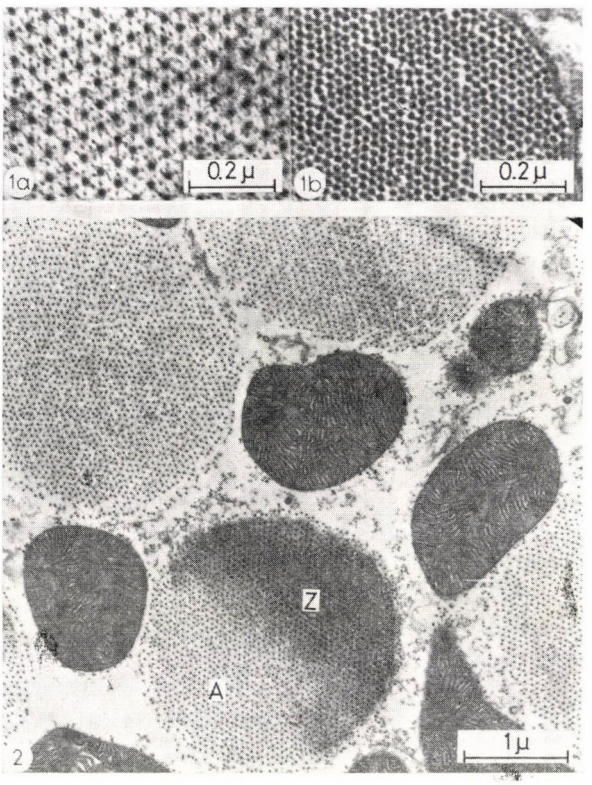

Fig. 1. Transverse sections of bee wing muscle fibrils. a: fixed in situ, the centre-to-centre distance of myosin filaments is about 500 Å. b: highly stretched. The lateral separation decreased to about its half in relation to Fig. 1a

Fig. 2. Transverse section of a bee wing muscle fixed in situ and contracted presumably by the effect of the fixative (in Fig. 5b a longitudinal section prepared from the same block will be shown). Note the dense mitochondria exhibiting a well preserved, normal structure

Fig. 2 represents myofibrils in cross-section taken from a muscle fixed in situ and presumably contracted by the effect of the fixative. This is indicated by the lack of I-bands. Since this muscle does not shorten considerably on physiological conditions (working nearly isometrically), the sarcomere length of the muscle contracted in situ cannot be very far from its sarcomere length corre-

sponding to the resting length (see Fig. 5b). The diameter of these fibrils is slightly more than 2.5 μ.

The diameter of fibrils of a highly stretched muscle originating from the same series of preparation and embedding and shown in Fig. 3 does not exceed 1.5 μ. This means an about four-fold decrease of the cross-sectional area. Accepting the constant-volume behaviour of this muscle a decrease of the cross-sectional

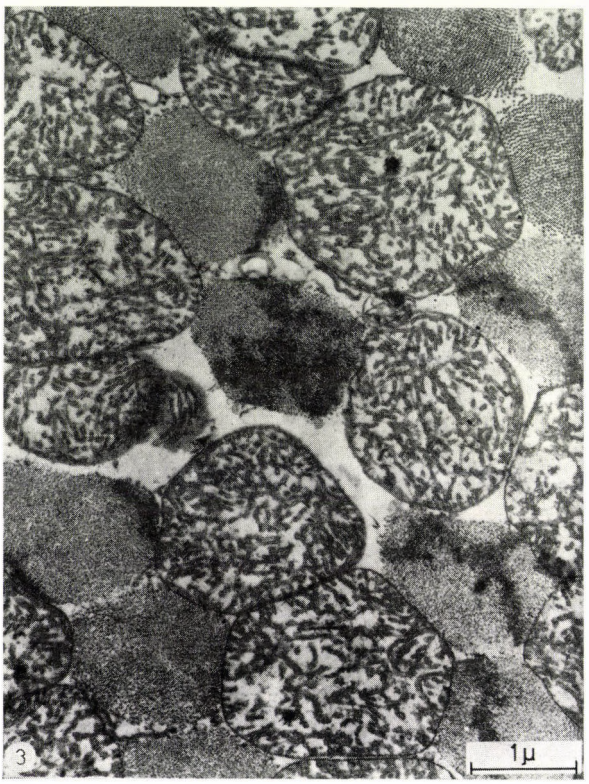

Fig. 3. Transverse section of a highly stretched bee wing muscle. Note the strong decrease of the cross-sectional area of myofibrils and the enormous expansion of the mitochondria

area like this would correspond to a fourfold elongation. Since the longest sarcomeres ever observed by us (Garamvölgyi, 1966) are not longer than 8.5–9 μ corresponding to an elongation slightly higher than threefold, it is obvious that even this extremely extensible muscle is incapable to reach a fourfold resting length.

Our assumption that the lattice spacing of this muscle does not follow the constancy of volume seems also confirmed by the size of the mitochondria. All mitochondria of the stretched muscle are enormously expanded indicating that the filament spacing is not decreased by a passive lateral compression caused by

the cell surface. In contrary, it seems that the expansion of mitochondria compensates for a decrease of the internal pressure caused by a volume decrease of myofibrils. The volume changes of mitochondria accompanied by a loosening of their structure cannot be the result of a bad fixation, for the mitochondria of unstretched muscles (Fig. 2) are dense, their tubules (or cristae) are intact, i.e. they seem rather well preserved.

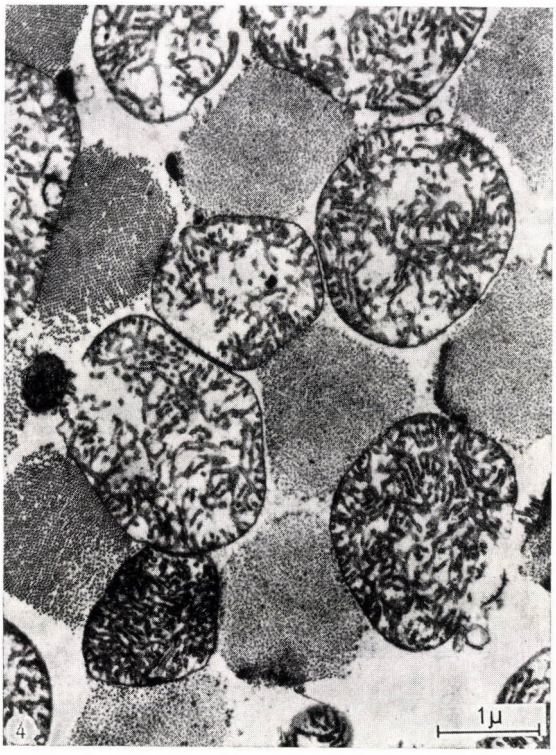

Fig. 4. Similar to Fig. 3 (in Fig. 5a a longitudinal section from the same block will be shown)

Fig. 4 shows a muscle consisting of myofibrils of diameter and lattice constant similar to that of Fig. 3. The sarcomere length corresponding to this lateral spacing can be established by preparing longitudinal sections from the same block. This is shown in Fig. 5a. The sarcomere length is about 5 μ, a value close to the end of the overlap. There are in fact only very short residual overlap zones, but the stretch of myosin filaments did not yet begin. This striation pattern corresponds to a length slightly less than the twofold resting length (Garamvölgyi, Belágyi, 1968; Garamvölgyi, 1969). The overwhelming majority of sarcomeres exhibited this length, there were only a few sarcomeres in which the stretch of myosin

filaments just began. The sarcomere shown in Fig. 5b has been prepared from the same block as shown in Fig. 2. The sarcomere length is about the half of that shown in Fig. 5a. As a consequence, in this muscle a twofold increase of the sarcomere length corresponds to a fourfold decrease of the cross-sectional area, i.e. about 100 per cent more than required by the constancy of muscle volume.

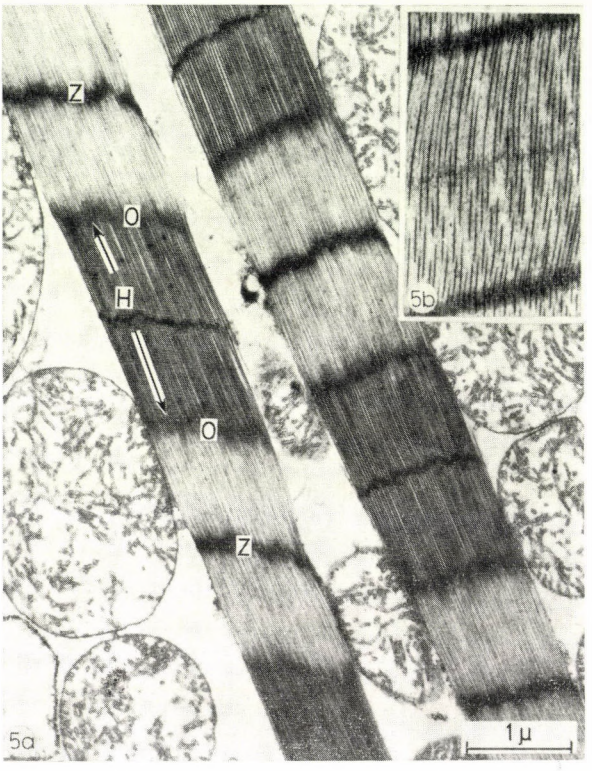

Fig. 5. Longitudinal sections, a: from the specimen shown in Fig. 4, S ~ 5.0 μ; b: from the specimen shown in Fig. 2, S ~ 2.6 μ

We regard this observation as a qualitative indication. A more precise and systematic determination of the filament spacing over the entire range of the sliding movement can be performed more appropriately by the method of X-ray diffraction. Nevertheless, it seems improbable that the constancy of volume should control the filament distance.

Discussion

The changes of the lateral separation of myofilaments have been systematically studied by Elliott and Rome (Elliott, Rome, 1967; Elliott, 1967, 1968; Rome, 1967, 1968). It has been stated that the lateral separation is controlled by

the pH, ionic concentration and length. In this connection one has to raise the question, what is the difference in principle between the constant volume-behaviour of vertebrate muscles (Huxley, 1952; Elliott et al., 1967) and the different behaviour of bee muscle?

Most probably it is the different lattice geometry which may explain this phenomenon. It is well known that in vertebrate muscles the actin filaments occupy a trigonal position in the centres of triangles formed by three myosin filaments, while in the insect wing muscle (and in some muscles of other invertebrates) the actin filaments are situated at mid-distance between two myosin filaments (Huxley, Hanson, 1960). If this assumption of ours is acceptable, it is evident that the physical forces acting between myosin and actin filaments are of particular importance from the point of view of filament spacing control.

It can hardly be accepted that there were effective forces acting between myosin filaments maintaining a regular hexagonal lattice, but no forces should possibly act between myosin and actin filaments being much closer to each other in all kinds of muscle than the distance between two myosin filaments. If the forces acting between myosin and actin filaments can influence the lateral spacing, the regulation of the latter can be easily interpreted by the changes of the degree of overlap. The forces acting between myosin filaments (both adhesive and repulsive) are more or less independent of the sarcomere length, since myosin filaments usually do not come out from their register considerably. Contrary to this, the forces acting between myosin and actin filaments obviously depend on the degree of overlap, i.e. on the presence or absence of actin filaments in the different zones of the A-band. It is evident that the resultant of the long-range forces will also be length-dependent.

At the length of the excised muscle (equilibrium length or slack length) the resultant of forces acting between filaments will be in the state of equilibrium with zero external stretch force. If the muscle is stretched, the axial stretch force may shift the balance of transverse forces in the direction of adhesive forces and this will decrease the lateral spacing of filaments. It is evident in this case that the changes of the lateral separation will be influenced by the relative position of the myosin and actin filaments and hence vertebrate and insect muscles may be expected to behave differently.

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Slow and Fast Muscle Cells in Human Striated Muscle

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Two different kinds of cells have been identified in normal human striated muscle. There are differences in the number of mitochondria as well as in the frequency of triads. The main difference is represented by the different width of the Z-lines. On the basis of comparative data this difference may be regarded as the structural equivalent of slow and fast muscles.

Introduction

The physiological difference of fast and slow muscles seems mainly determined by the innervation and partly by factors outside the contractile apparatus, i.e. by the different amount of myoglobin and by differences in the enzymic system of cell metabolism. Nevertheless, the physiological properties seem to have their equivalent on the level of the contractile apparatus, too. Earlier it was stated that in slow muscles the cross-striation is more expressed, whilst in fast muscles the myofibrillar structure is much more clearly visible. Working with the electron microscope distinctions like these are totally useless, since we know that all vertebrate striated muscles consist of myofibrils and all myofibrils are cross-striated.

More reliable differences could have been established by studying transverse sections. According to Krüger (1929) the myofibrils of fast muscle cells are well-separated ("Fibrillenstruktur"), while in slow muscles the myofibrils usually melt together forming large cross-sectional areas ("Felderstruktur"). This statement was later confirmed by Hess (1960), too. Peachey and Huxley (1962) revealed the relationship between the physiological character and the morphological differences by measuring the speed of contraction visually before fixation of individual fibres of frog muscles. Thus slow and fast muscle fibres of the frog could have been identified as morphologically different "Felderstruktur" and "Fibrillenstruktur" fibres.

On this basis the structural characteristics of the two kinds of cells of frog muscle have been studied by Peachey and Huxley (1962) and later in a more detailed form by Page (1965). The most important observation concerned the amount of the sarcoplasmic reticulum. In general this is larger in fast cells and, as

a consequence, the fibrils are perfectly separated by the sarcoplasmic reticulum. Contrary to this, in slow fibres the smaller amount of the sarcoplasmic reticulum does not prevent the fibrils from melting together in a "Felderstruktur" form. In total agreement with this statement Page (1965) found triads in fast cells at virtually all Z-lines, while in the slow cells there were triads at every fifth or sixth Z-line only.

Very important differences were found also in the structure of myofibrils. In the fast cells the Z-lines are thin and straight and their tetragonal array is rather perfect. There is a regular M-line in each sarcomere. Contrary to this, in slow fibres the Z-lines are much thicker and zig-zag-shaped, their cross-sectional structure is less regular. The M-lines are missing. The same differences were observed in the two kinds of cells in muscles of the garter snake (Hess, 1965) and in the extraocular muscle of the rabbit (Cheng-Minoda et al., 1968).

Page (1969) studied later the anterior and posterior latissimus dorsi muscles of the chicken from the same point of view. Contrary to frog muscle (and even to chicken breast muscle) the triads are located near the A – I junctions. Nevertheless, the amount of the sarcoplasmic reticulum was found different analogously to frog muscle. The Z-lines were found more than twice as thick in the slow than in the fast muscle. In relation to the M-lines no difference exists, they are present in both kinds of cells.

Different rat muscles were recently studied by Gauthier (1969). As the most obvious difference she also pointed out the width of the Z-line. In these muscles, too, there is an M-line in all sarcomeres. She believed to have found a third intermediate type of muscle, just on the basis of the different width of the Z-line. According to her it is the size of the Z-line which may be regarded as the most reliable criterion of the physiological character.

Though in this paper we compare only the data concerning vertebrate muscles, it is necessary to mention the works dealing with invertebrate muscles of different speed (e.g. Auber, 1967; Reger, 1967).

Materials and methods

Samples of normal human muscle (m. obliquus abdominis) were excised from adult individuals subject to abdominal operations. The muscle columns measuring about 2 cm in length and 2 – 3 mm in diameter were fixed at constant length immediately after their excision. The range of length extended from the equilibrium length to an elongation of about 100 per cent. The prefixation was performed in 2.5 per cent buffered glutaraldehyde for 3 hours. After this the muscles have been cut and dissected into small pieces. All samples were taken from the central parts of the columns, where the silken thread used for mounting the muscles could not cause any injury. The samples were then postfixed in 1 per cent osmium tetroxide for 1 hour. For embedding we used Durcupan-Fluka ACM araldite. The sectioning was carried out by an LKB "Ultrotome III" ultramicrotome. The sections were stained by uranyl acetate and lead citrate. The specimens were studied in a WF SEM-3 electron microscope.

Observations

a) *Myofibrils*

The main difference in the structure of myofibrils of the two different kinds of cells can be observed in the size of the Z-lines (Fig. 1). In one kind of cells (Fig. 2) the Z-lines measure about 400 Å. As compared with frog and chicken muscles (Page, 1965, 1969) these cells may be considered as fast cells. In the second kind of cells (Fig. 3) the width of the Z-line is about 1000 Å. Most probably these are slow muscle cells. The striking difference in the size of the Z-lines can be demonstrated also at higher magnification (Fig. 4). Contrary to frog

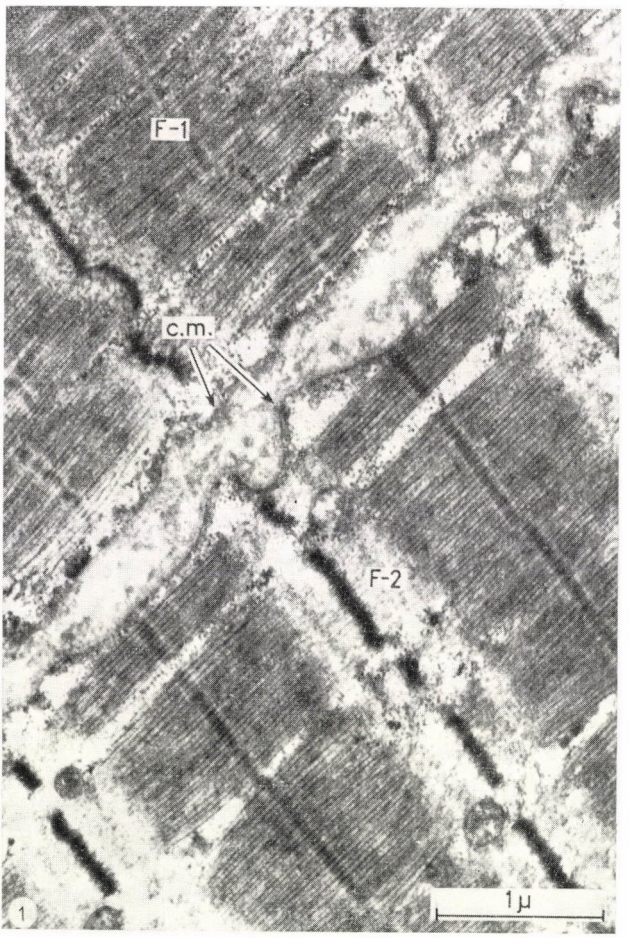

Fig. 1. Longitudinal section showing two adjacent cells of human oblique abdominal muscle. c.m.: cell membranes; F-1: a fast fibre, exhibiting relatively narrow Z-lines; F-2: a slow fibre the Z-lines of which are considerably thicker

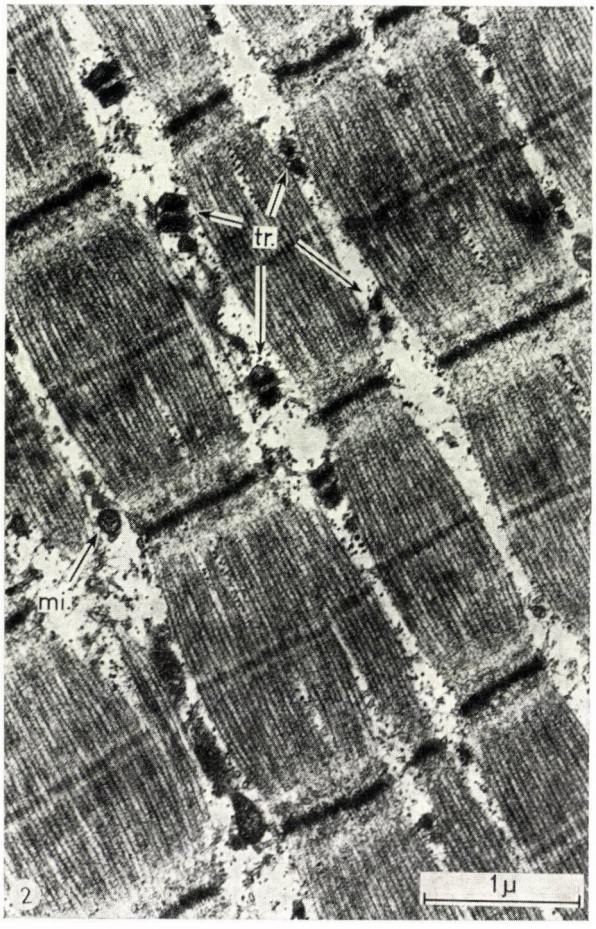

Fig. 2. Longitudinal section of a fast fibre from human m. obl. abd. fixed near its equilibrium length. tr: triads. There are only a few mitochondria (mi). The Z-lines are narrow

muscle (Page, 1965) the Z-lines of slow fibres seem perfectly straight. Like in the two kinds of fibres of chicken (Page, 1969) and rat muscle (Gauthier, 1969) there are also in human muscle M-lines in both slow and fast cells. Both kinds of cells seem to be subdivided into regular myofibrils and no reliable distinction can be made on the basis of "Fibrillenstruktur" and "Felderstruktur", respectively.

b) *Mitochondria*

In human muscles studied in longitudinal orientation two small mitochondria can be usually observed at the level of the I-band, symmetrically in relation to the Z-lines (Fig. 3). In fact these are profiles of long, thread-like mito-

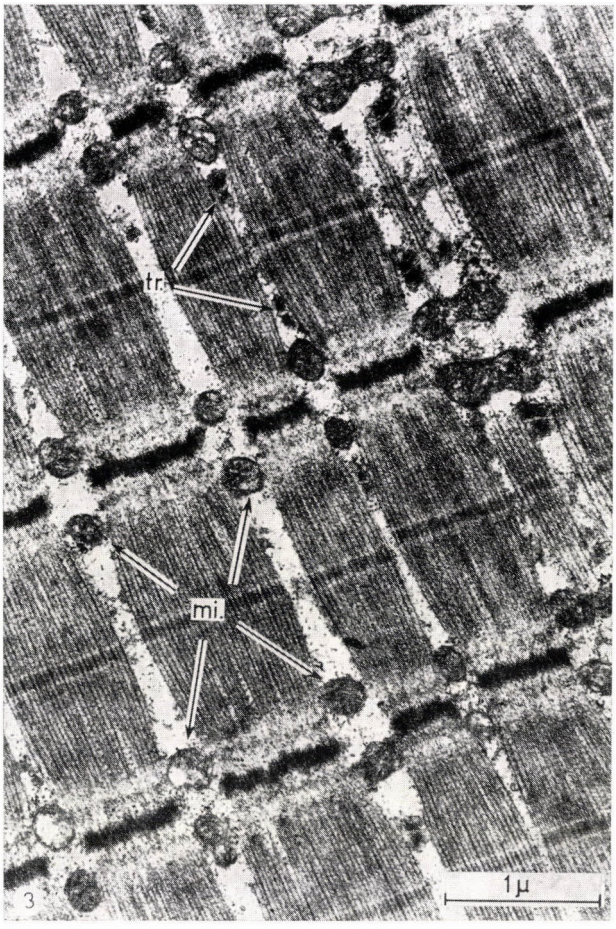

Fig. 3. Longitudinal section of a slow fibre of the same muscle as shown in Fig. 2. The Z-lines are more than twice as thick. Note the regular occurrence of mitochondria at the level of virtually all I-bands

chondria oriented perpendicularly to the fibre axis. The occurrence of mitochondria is much more frequent in slow (Fig. 3) than in fast fibres (Fig. 2). This fact confirms our assumption made on the basis of the myofibrillar structure.

It is very probably the higher number of the mitochondria and their rather regular arrangement which make the formation of the "Felderstruktur" in slow human muscle impossible. At low magnifications slow cells exhibit an even more expressed fibrillar appearance, due to the numerous mitochondria situated regularly and being present at virtually all I-bands. Just by the lack of regular mitochondrial arrangement in fast fibres the separation of myofibrils is even less complete.

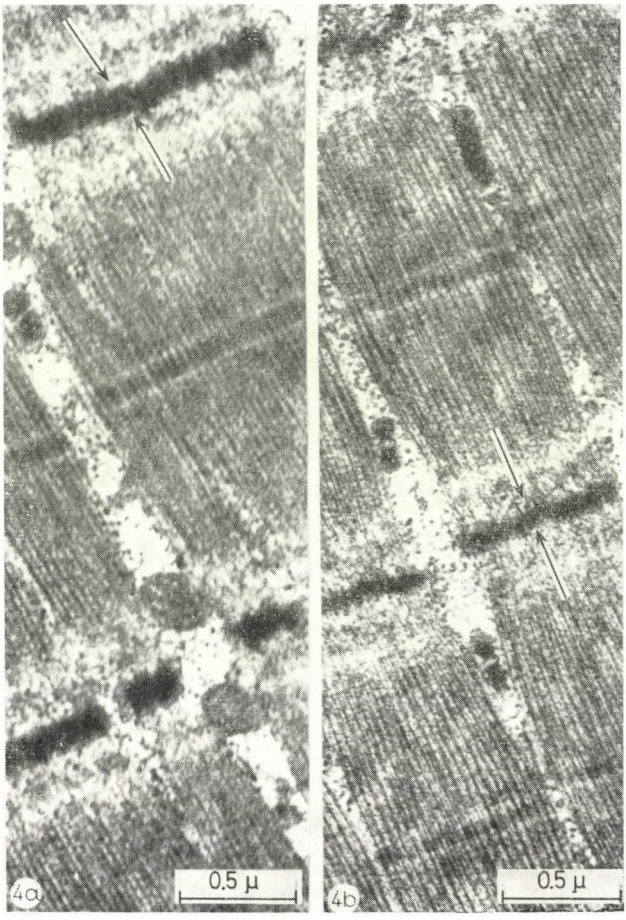

Fig. 4. Longitudinal section of a slow (a) and of a fast (b) fibre. Compare the width of the Z-lines

c) *Sarcoplasmic reticulum, T-system, triads*

Typical triads uniting the terminal cisternae with elements of the T-system may be observed in both kinds of cells. The terminal cisternae are rather electron-dense containing granular substance (Figs 2, 3 and 5). In fast cells triads seem more frequent. This is very probably due to the longer terminal cisternae in this kind of cell. Sometimes triads happen to be cut tangentially between two myofibrils (Fig. 5). In such cases triads appear as long tripartite ribbons surrounding the myofibrils at mid-distance between Z- and M-lines, i.e. close to the A–I junctions. Tangential (from the point of view of triads "longitudinal") sections of triads as shown in Fig. 5 appear usually in fast cells only. This is why the prob-

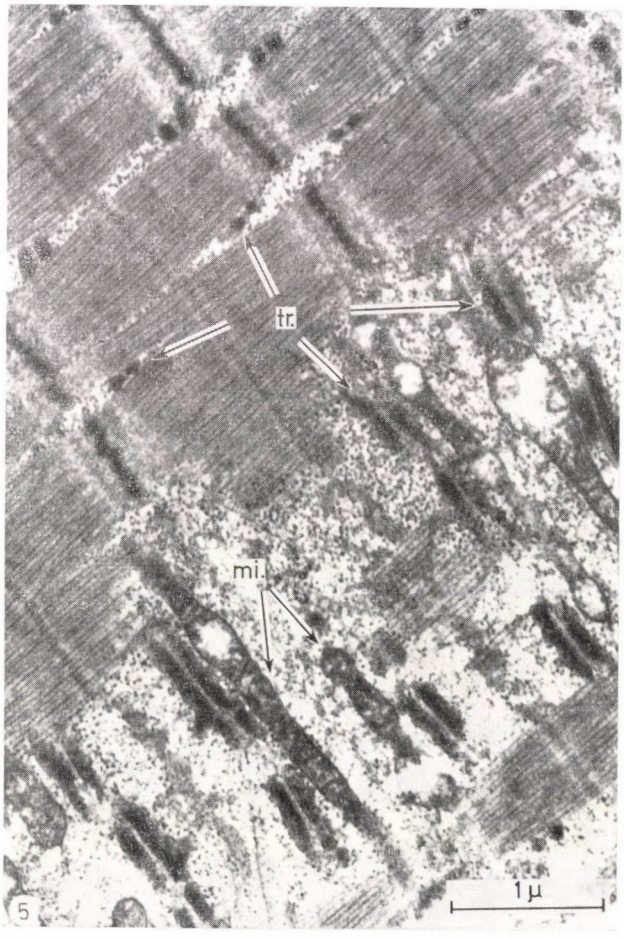

Fig. 5. Longitudinal section of a fast fibre exhibiting numerous triads some of which are cut "longitudinally" (i.e. tangentially)

ability of their presence within a given section thickness is higher in fast than in slow muscle.

In general the entire amount of the sarcoplasmic reticulum in human muscles seems rather small as compared with frog muscle. The terminal cisternae are much smaller and only very few longitudinal tubuli can be identified. So this fact may contribute to the lack of sharp differences in the size of the myofibrils.

Discussion

On the basis of our observations we agree with Gauthier (1969) according to whom it is the size of the Z-line which may be considered as the most reliable equivalent of physiological properties. The distinction "Fibrillenstruktur"—

"Felderstruktur" can hardly be accepted as reliable. It does not mean, of course, that the two different cross-sectional arrangements were not an indication of the slow or fast character of cells of other muscles, but in this muscle extrafibrillar structures, particularly mitochondria, can prevent the formation of a "Felderstruktur".

In our opinion the two kinds of physiologically different cells can be distinguished by careful comparative analysis. The most useful indication appears to be the size of the Z-line, combined with an estimation of the relative number of mitochondria.

The most important application of the distinction of the two kinds of muscle cells in human muscle is the clinical field. It is well known from the biochemistry and pathology of muscle that slow and fast cells react differently to metabolic diseases. It is therefore absolutely necessary to study both kinds of cells simultaneously in all cases of muscle diseases and injuries.

The author is deeply indebted to Dr. I. Löblovics for carefully prepared human surgical samples as well as for many advices. He thanks also Miss Margit Stark for her reliable technical assistance.

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Study of the UV Effect on MS2 Phages*

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The effect of $\lambda = 254$ nm UV light on the plaque forming activity of MS2 phages has been examined. It has been established that the dose-effect curve similar to that of the T7 phages is of the one hit type and, when seen from above, its shape is concave. An attempt was made to analyse the dose-effect curve with our radiation kinetic model developed for the T7 phages. The deviation of the dose-effect curve from that of the T7 phages was assumed to be brought about by the induction of different photoproducts of the DNA and RNA bases: upon the effect of UV irradiation uracil may undergo, besides dimerization, hydration, too.

From the initial phase of the MS2 dose-effect curve and from the comparison of the T7 and MS2 dose-effect curves the probability of phage inactivation due to dimer formation and to hydration has been determined and found to be about $2 \cdot 10^{-4}$ and $1 \cdot 10^{-4}$, respectively. The former value is similar to the probability obtained for T7 phages.

Introduction

In previous experiments we have established the UV inactivation dose-effect curve of T7 phages and have given it a quantitative interpretation (Rontó et al., 1967). On the basis of our radiation-kinetic model and the molecular-biological data concerning the T7 phages (Summers, Szybalski, 1968; Studier, Maizel, 1969; Summers, 1970) we have arrived at the conclusion that structural injury of the DNA molecule appears functionally at the level of transcription (Rontó, Tarján, 1971). In comparison of the radiation injury of transcription and translation it is the study of the UV injury of MS2 phages which seems to be of interest. The MS2 phages contain a single stranded RNA possessing a messenger function as well (Haruna, Spiegelman 1965; Sinsheimer, 1964; Weismann, 1967; Grubman, Nakada Dai, 1969); thus, the UV inactivation of the MS2 phages has a connection with the radiation sensitivity of translation.

The UV inactivation dose-effect curve of MS2 is known from the work of Rappaport (1965). In his opinion the number of the active phages diminishes exponentially with the increase of the dose. In our opinion the dose-effect curve established by Rappaport can be applied only for the rough estimation of ten-

* Based on a paper read at the VIth Congress of the Hungarian Biophysical Society.

dencies as the fine analysis of the initial phase was not carried out. In the present work we made an attempt to determine the exact inactivation dose-effect curve of the MS2 phage. On the basis of our results we have examined whether our radiation kinetic model for T7 phages can be applied for MS2 phages as well. In addition, we have completed the model with supplementary data to enable us to interpret the divergence experienced in the course of the radiation studies of the MS2 phages.

Methods

The experiments were performed on MS2 phages and *E. coli* C3000 hosts, under conditions similar to those used in our previous studies with the T7 phages (Rontó, Tarján, 1967).

The concentration of the phages not treated with UV light (controls) was determined as follows. In some experiments both plate-pouring and incubation of the phage-bacterium complex at 37 °C were carried out in total darkness. In other cases plate pouring was performed in normal room light and also the phage-bacterium complexes received normal room light during the first burst time (for about 30 minutes). In the third series of the experiments an extra light, too, was applied: after plate-pouring, the phage-bacterium complexes were placed at a distance of about 1/2 to 1 m from the 300 W electric bulb and were illuminated similarly for 30 minutes. (During light treatment the Petri dishes did not get warm.)

To study photoreactivation we took parallel samples from the irradiated phage-suspension on each occasion. One of the samples was incubated in darkness, the other was illuminated, similarly to the control (the temperature of the two samples was always identical).

Results and discussion

1. Figure 1 shows the initial phase of the MS2 dose-effect curve. In connection with the curve we should like to point out the following:

a) According to the data in the literature depending on the circumstances, only 5 to 50 per cent of the RNA phages have a plaque-forming ability (Weismann, 1967; Lodish, 1968). For example, the exposure to light or heat treatment of the phage-bacterium complex (see Methods) may influence up to ± 10 per cent the number of plaque-forming phages. Thus, by strict standardization of our experimental conditions, we obtained results with the relatively small experimental errors shown on the Figure.

b) The initial slope is less than zero proving that the curve is of the one hit type.

c) Seen from above the curve is concave, that is, towards higher doses its slope decreases. Consequently, similarly to T7 in case of the RNA containing phages, the reactivation of the UV light injury must be taken into account as well.

d) According to our measurements, within a fourfold intensity range, the shape of the curve is unrelated to the intensity of the UV light and depends only on the dose.

2. Of the various reactivations multiplicity reactivation, host cell reactivation, enzymatic photoreactivation and direct photoreversion must be taken into consideration. Our experimental conditions exclude multiplicity reactivation. As

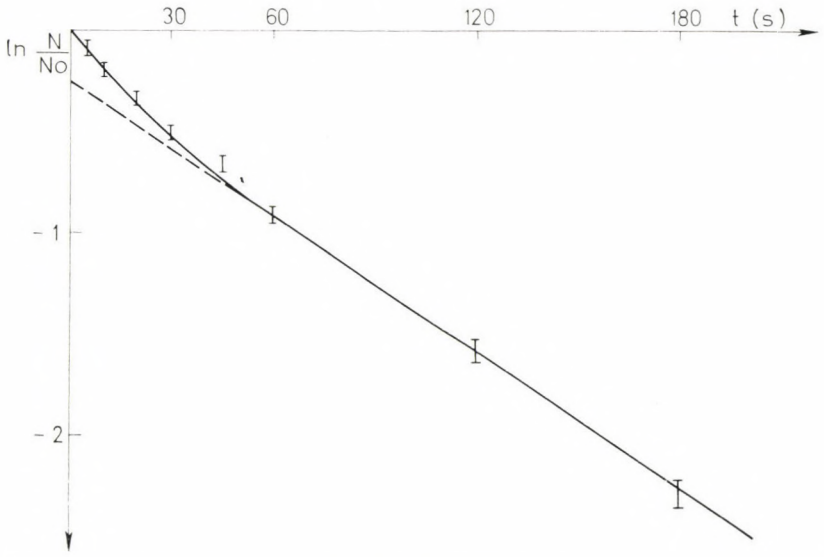

Fig. 1. The UV inactivation dose-effect curve of MS2 phage. The natural logarithm of the survival rate is plotted against time

according to the experiences until now the substrate of the host cell reactivation is a double stranded DNA, it can be assumed that, in the case of MS2 phages, no possibility exists for reactivation. Enzymatic photoreactivation was established in the case of some RNA viruses (McLaren, Shugar, 1964; Dertinger, Jung, 1969). To elucidate whether this phenomenon may occur with MS2 phages the UV dose-effect curves were measured in darkness and under exposure to light. In our experiences the two curves were identical. Consequently, we can establish the fact that in our system radiation injury is not modified by enzymatic photoreactivation either.

We assume that, in accordance with our experiences obtained on T7 phages (Rontó et al., 1967), the smaller terminal slope of the dose-effect curve is due to the direct reverting effect of the UV photons. However, the possibility of activation of the initially inactive phages of the lysate by the UV photon cannot be excluded at present.

3. In order to compare the injury of the T7 and MS2 phages we show in Figure 2 the natural logarithms of the survival rates of the two phage types in

the function of the average number of photons absorbed by one phage. The incident light intensity was $1 \cdot 10^{14}$ photons/cm^2 × s in both cases. In the case of T7 phages we have directly determined, with the help of the absorption constant, the average number of photons absorbed by one phage in one second (m_{T7}) and found it to be $m_{T7} \approx 1.5 \cdot 10^3$ photons/phages × s. In the case of the MS2 phages the absorption constant can be established only approximately as the phage

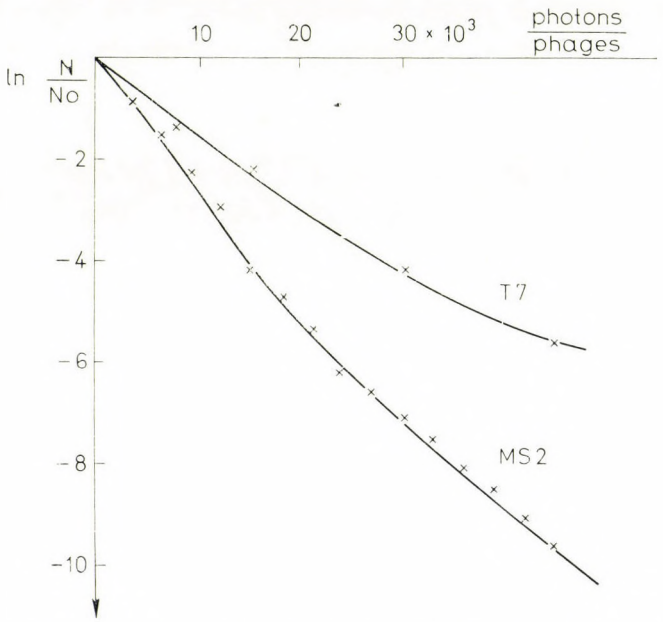

Fig. 2. The comparison of the UV inactivation dose-effect curves of MS2 and T7 phages. The natural logarithm of the survival rates is plotted against the average number of photons absorbed by one phage. (To avoid confusion only one part of the data of Fig. 1 belonging to the initial part of the curves is shown)

ysate is not pure and concentrate enough. Because of this the number of photons absorbed by one phage in one second (m_{MS2}) was estimated on the basis of the knowledge that one MS2 contains about thirty times less nucleotide bases than one T7. According to our estimation $m_{MS2} \approx 50$ photons/phages × s.

Figure 2 reveals that the shape of the two curves is divergent, the MS2 dose effect-curve being steeper than in the case of T7. We assumed that this deviation was brought about by the difference of the meanly UV sensitive pyrimidine bases, i.e. cytosine in the T7 DNA and uracil in the MS2 RNA. It is well known from in vitro experiments (McLaren, Shugar, 1964; Setlow, 1966; Dellweg, 1968) that UV light induces the development of dimers in cytosine and the development of both dimers and hydrated photoproducts in the case of uracil. Both types of dimers can be reverted by a new UV photon, but this phenomenon does not refer to the hydrated photoproducts (McLaren, Shugar, 1964; Setlow,

1966). We think that both the dimers and the hydrated photoproducts cause phage inactivation. In the case of MS2 a superposition of these two effects occurs, thus, the dose-effect curve is steeper.

We should like to emphasize another fact. If the doses shown in Figure 2 are doubled, the T7 dose-effect curve already tends towards saturation (Rontó, Tarján, 1967); the same is not true for MS2. Also this deviation is thought to be due to the hydrated photoproducts.

4. On the basis of the facts described in Point 3, some quantitative conclusions can be drawn. At the beginning of the UV irradiation ($t = 0$ time, when there is no UV-damaged phage in the phage suspension) the number of active phages (N_0) can only decrease as a result of photon absorption. We do not consider the possibility that formerly inactive phages of the lysate may be activated by UV light. The decrease in number of active phages may be due to the production of dimers and hydrated photoproducts. The probability of the production of the former is α_{MS2}, and that of the latter is κ. Therefore

$$\frac{1}{N_0}\left[\frac{dN}{dt}\right]_{t=0} = -(\alpha_{MS2} + \kappa)\, m_{MS2}$$

and knowing m_{MS2} from the initial slope of the dose-effect curve, $\alpha_{MS2} + \kappa$ can be determined. This value, that is the probability of inactivation of one phage by one absorbed photon, was found to be $3 \cdot 10^{-4}$.

The value κ could be calculated from the terminal slope which can be determined at high doses. Thus $\kappa \approx 1 \cdot 10^{-4}$ and $\alpha_{MS2} \approx 2 \cdot 10^{-4}$; this value is in good accordance with the α value obtained previously for the inactivation probability of T7 phages (Rontó et al., 1967).

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Effect of ^{60}Co-γ-Rays on the Infrared Spectra of DNA

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High polymer calf thymus DNA solutions have been investigated by infrared spectroscopy between 800—1700 cm^{-1}.

^{60}Co-γ-irradiation ($\sim 10^4$ kR) caused only a minor alteration in the infrared spectra, mainly due to the damage of different bases. Higher irradiation doses ($\sim 10^5$ kR) increased the spectral changes at wavenumbers characteristic of certain bases. However those spectral parts characterizing the number of base-pairs remained practically unchanged after doses that produced degradation well detectable by viscosity measurements.

Introduction

The secondary structure of macromolecules can be influenced by several physical and chemical methods. As the macromolecular structure and function have close interconnections, a great number of studies have been carried out to obtain more information in this field.

Ionizing radiation proved to be a useful tool in studying the molecular structure of DNA and RNA. On the other hand radiation sensitivity of DNA and RNA have been playing a central role in the radiation biology since the early fifties.

Several authors investigated the radiation sensitivity of H-bridges of nucleic acids (Alexander, Lett, 1960; Emmerson et al., 1960; Hagen, 1964) and the viscosity of native and radio-denatured DNA. Since Eigner and Doty (1965) reported the sedimentation constant to be less dependent on the shape of the DNA molecules than is the viscosity, further investigations were carried out on the sedimentation patterns of irradiated DNA (Hagen, 1967).

Other research workers did not observe significant changes in the physical properties of DNA molecules even after X-ray irradiation with 10 kR (Freifelder, 1966; Gupta, Mitra, 1970). Recently Fritzsche presented evidence that thermal denaturation of highly concentrated DNA solutions in ^2H_2O can be followed by infrared spectroscopy (Fritzsche, 1971). As is known the infrared spectra of nucleic acids depend mainly on the number and quality of base pairs (Tsuboi et al., 1968; Tsuboi, 1970; Cotter, Gratzer, 1969).

In the present paper we studied the question how the radiation damage of concentrated high polymer DNA solutions could be followed by infrared spectroscopy.

Materials and methods

High polymer calf thymus DNA was purchased from Sigma Chemical Company. 86 °C was determined as T_m point for DNA. Different amounts of DNA were dissolved in 99.8 per cent ^2H_2O or in H_2O. Dissolved samples were stored in sealed test tubes at 0–5 °C. Immediately before experiments, samples were tempered at 40 °C and divided into two parts. Sodium dodecylsulphate (SDS) was added to one part of the samples to obtain 0.5 per cent (w/v) saturation.

Both control and SDS containing samples were irradiated by ^{60}Co-γ-rays with a 2470 R/min dose rate at 20 °C, without filter.

Infrared spectra of irradiated and native samples were investigated in a Zeiss-UR-10 double beam spectrophotometer. Samples were prepared in KRS-5 cells having a thickness $d = 0.06 \pm 0.005$ mm. The samples usually contained 25 mg/ml of DNA and 0.01 M NaCl in ^2H_2O or in H_2O. The solvent constituents were put into the reference cell. Since the infrared spectra of H_2O shadowed a greater part of DNA's infrared peaks, the ^2H_2O was preferred as solvent. The $\frac{I}{I_0} \cdot 100$ values were determined by "base-line" techniques (Holly, Sohár, 1968). Silicagel was put into the cell holder of the spectrophotometer to keep water saturation low during the experiments. The pH of solutions was kept at 7.2. Viscosity was determined by Ostwald viscometer.

Results

Since the formation of infrared spectra of nucleic acids depends on the paired bases, attention was mainly paid to the 800–1700 cm^{-1} wavenumber interval. Table 1 shows the wavenumbers characteristic of the AT and GC base-pairs. The characteristic wavenumbers of base-pairs were determined in solid phase, using AgCl plates (Tsuboi et al., 1968).

Fig. 1 demonstrates the infrared spectra of calf thymus DNA dissolved in 0.01 M NaCl and ^2H_2O.

Table 2 and Fig. 2 show the effect of ^{60}Co-γ-irradiation on the $\frac{I}{I_0} \cdot 100$ values – determined by base-line techniques – belonging to different constituents of DNA. Table 2 points also to the character of infrared absorption at different wavenumbers.

As shown in Fig. 2 the peaks characteristic of the AT and GC base-pairs did not express dose-dependency concerning the relative transmittance. A slight flattening and broadening appeared at 1476 cm^{-1} so that this peak shifted to 1480 cm^{-1}.

Table 1

Wavenumber cm^{-1}	Groups	Character of groups
967	O	s
1017	O	m
1050	O	s
1087	O	s
1153	AT	w
1302	GC	m
1334	AT	w
1377	GC	w
1458	AT	w
1476	AT	m
1576	GC	s
1618	AT	s
1623	GC	m
1676	GC	s

Symbols: O = other; AT = Adenine-Thymine, GC = Guanine-Cytosine } base-pairs; s = strong, m = medium, w = weak

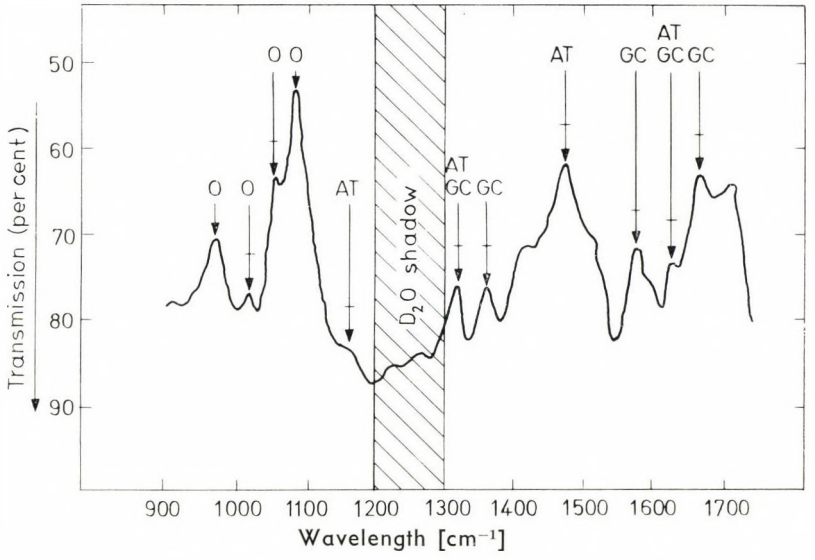

Fig. 1. Infrared spectrum of native DNA solution

Data of SDS treated and irradiated samples are summarized in Table 3 and Fig. 3.

In the presence of SDS transmission values of all peaks were increased by irradiation compared with the SDS treated control. Marked changes were found

Table 2

Dose [kR]	Wavenumber [cm^{-1}]							
	967 O–s*	1050 O–s	1087 O–s	1153 AT–w	1320 AT–w GC–m	1476 AT–w	1576 GC–s	1676 GC–s
0	84.81	79.26	66.26	97.63	89.15	77.77	89.02	79.26
28	87.35	80.68	71.60	97.93	91.11	81.39	95.23	80.35
56	96.05	76.13	65.16	97.77	90.24	82.05	91.25	77.65
84	87.50	80.00	67.50	96.38	92.20	78.37	93.33	81.55
112	91.76	81.80	63.63	93.33	93.97	74.35	92.50	80.75

* for the symbols see Table 1

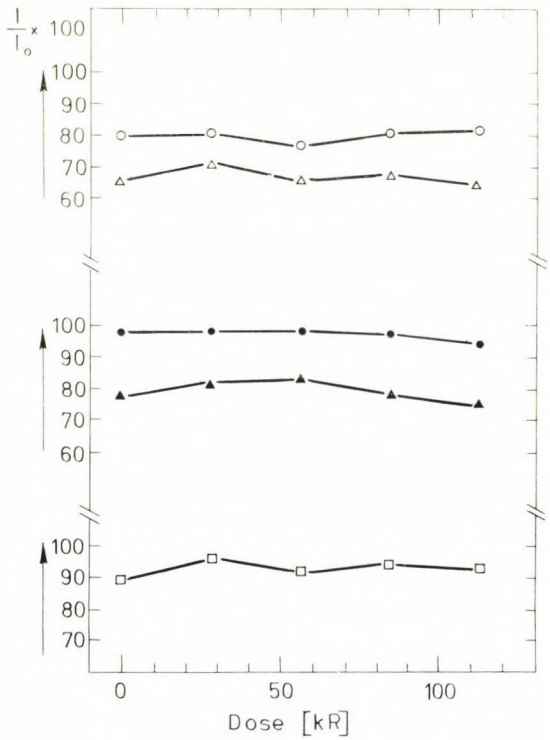

Fig. 2. $\frac{I}{I_0} \cdot 100$ values of DNA in ^2H_2O solution plotted against the irradiation dose. ○——○ 1050 cm^{-1} other peak. △——△ 1087 cm^{-1} other peak. □——□ 1576 cm^{-1} GC peak. ●——● 1153 cm^{-1} AT peak. ▲——▲ 1476 cm^{-1} AT peak

around 1153 cm^{-1}, 1476 cm^{-1}, and 1576 cm^{-1}. These peaks belonged to AT and GC base-pairs as well. The highest transmittance increase was observed at 1476 cm^{-1}.

Table 3

Dose [kR]	Wavenumber [cm^{-1}]							
	967 O−s+	1050 O−s	1087 O−s	1153 O−s	1320 AT−w GC−m	1476 AT−m	1576 GC−s	1676 GC−s
0	89.00	80.00	60.84	88.23	92.15	64.28	68.63	86.95
28	89.28	81.81	64.40	93.62	98.38	76.66	88.33	91.66
56	92.06	83.87	70.96	96.77	93.05	81.69	88.57	94.28
112	92.50	85.00	73.42	98.43	96.38	98.00	92.50	93.67

* for the symbols see Table 1

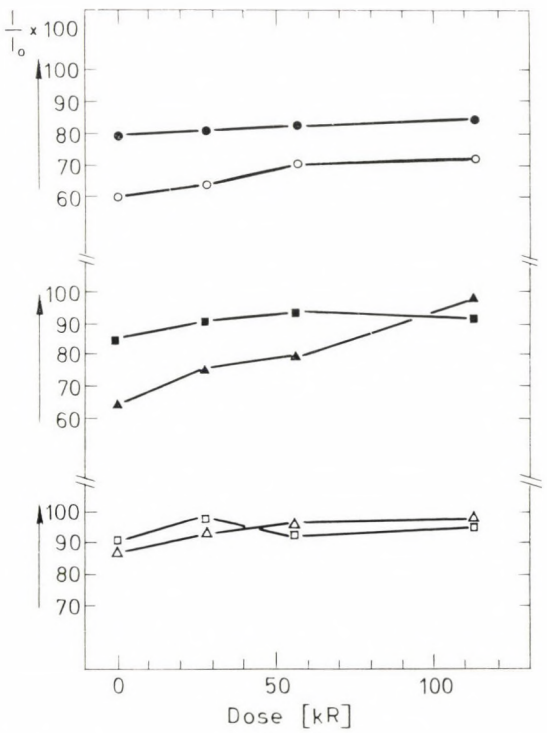

Fig. 3. $\frac{I}{I_0} \cdot 100$ values of the SDS-treated DNA in ^2H_2O solution plotted against the irradiation dose. ● ——— ● 1050 cm^{-1} other peak. ○ ——— ○ 1087 cm^{-1} other peak. △ ——— △ 1153 cm^{-1} AT peak. □ ——— □ 1320 cm^{-1} ATGC peak. ▲ ——— ▲ 1476 cm^{-1} AT peak. ■ ——— ■ 1676 cm^{-1} GC peak

Experiments determining the relative viscosity of native, SDS-treated and irradiated samples have been carried out parallel with the infrared spectroscopy. Viscosity of samples, after tenfold dilution with H_2O, have been determined by

Ostwald viscometer at 20 ± 0.5 °C. Relative viscosity values plotted against the irradiation dose are shown in Fig. 4.

The relative viscosity values of native DNA decreased in an exponential manner with the radiation dose. Pretreatment of DNA with SDS caused a marked change in the shape of the dose-response curve.

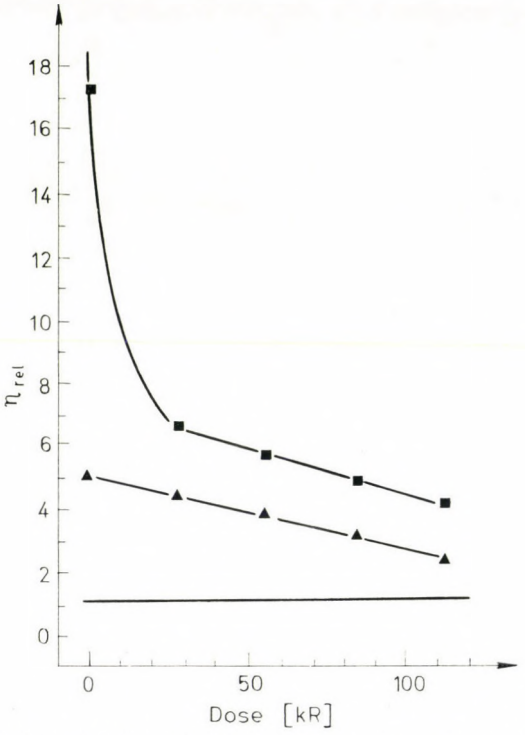

Fig. 4. ——— relative viscosity of ^2H_2O. ■ ——— ■ relative viscosity of DNA. ▲ ——— ▲ relative viscosity of SDS-treated DNA

Discussion

The energy of ^{60}Co-γ-rays (1.17 MeV) can destroy any of the P−O, C−O or C−C bonds as well as the much weaker H−H bonds along the double helical DNA. The results of viscosity determinations suggest that double strand breaks are in front of the radiation damage. Double strand breaks can take place between two complementary base pairs, however, also secondary double strand breaks are caused by simultaneous single breaks in the two strands which are not farther apart than 10 base pairs. According to Hagen (1967) the H-bridges are not strong enough to keep the two strands together in the above mentioned case.

The surprisingly slight changes caused in the infrared spectra by rather high doses of irradiation suggest that direct damages of bases are far less significant in radio-denaturation of the DNA than are strand breaks. The relatively greatest alteration observed around 1476 cm^{-1} in the presence of SDS showed a little higher sensitivity of AT base pairs when compared with the GC pairs.

Experimental results obtained in the presence of SDS point to the importance of H-bonds in the formation of the infrared absorption of nucleic acids. On the other hand the infrared and viscosity measurements refer to a reversibility of the post-irradiation H-bond breaks. The structure of DNA is far more complicated than that of small organic molecules. This is the reason why the changes in infrared absorption have not enabled us to specify exactly the alterations caused by irradiation or detergent. Further experimentation may bring us closer to the understanding of DNA's structure in solution.

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Dose Rate Stability of a Biological Irradiation Facility at a Thermal Reactor

(Short Communication)

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(Received December 9, 1971)

The dose rate stability of a biological irradiation facility operated at a thermal reactor is investigated. According to the present measurements it is better than ± 4 per cent, i.e. quite satisfactory for biological experiments.

A biological irradiation facility has been built in a horizontal channel of a thermal (WWR-Sm-type) reactor of the Central Research Institute for Physics, Budapest (Makra et al., 1970; Zaránd et al., 1971). The reactor is operated in 115 or 53 hour cycles at 3—5 MW thermal power ($2 \cdot \Delta W/W < 0.1$). The number of fuel elements must be altered periodically every 3 to 6 months because of burning out. It was the aim of the present work to investigate the dose rate stability of the facility throughout these periods, except the first day of a cycle on which no irradiation is made. The measurements were carried out with sulphur pellets and a G—M counter.

For a given core configuration the neutron spectrum may be assumed to remain unchanged during a 3 months period and the neutron dose rate can be monitored with sulphur pellets. The Φ_s values are calculated ($E_{\text{eff}} = 3$ MeV and $\sigma_{\text{eff}} = 300$ mb) from the sulphur activities. The neutron + gamma dose rate was monitored by means of a G—M counter built into the shield at a distance of 40 cm behind the irradiation cavity. The system was checked with a UO_2 standard. The standard error of the counting rate was 1.5 per cent over a day and 2.5 per cent over a 3 months period. The linearity of the monitor was determined by simultaneous measurements with G—M counter and S pellets while the reactor was operated at different power levels (Fig. 1, crosses) and may be considered satisfactory.

Table 1 summarizes the results of these measurements. The thickness of the Be reflector was practically unchanged during these measurements, so that every line refers to a different core configuration; as can be seen, the counting-rates vary with the configuration. The standard errors of the G—M counter readings are, however, in very good agreement with each other. The results assure the reproducibility of irradiations controlled with a switch clock.

The reading of the monitor is

$$R = a_1 D_n + a_2 D_{\text{gamma}} = D_n(a_1 + a_2 D_{\text{gamma}}/D_n) = \Phi_S \cdot a_3 (a_1 + a_2 D_{\text{gamma}}/D_n) \quad (1)$$

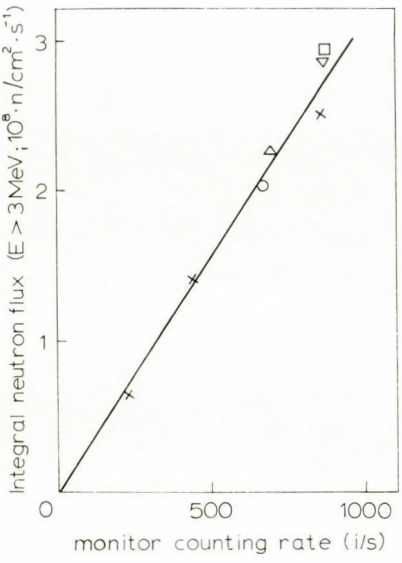

Fig. 1. Fast neutron flux vs. monitor counting rate (filter: 70 mm Bi + 10 mm B_4C)

Table 1

Monitor counting rate and fast neutron flux for different core configurations

Filter mm / Core configurations	70 Bi 10 B_4C			145 Bi 10 B_4C	
	I (i/s)	$\Delta I/I$	Φ_s(n · cm^{-2} · s^{-1})	I (i/s)	$\Delta I/I$
No. 1	694	—	$2.28 \cdot 10^8$	290	—
No. 2	850	$3.54 \cdot 10^{-2}$	$2.77 \cdot 10^8$	358	$3.6 \cdot 10^{-2}$
No. 3	867	$3.44 \cdot 10^{-2}$	$2.95 \cdot 10^8$	386	$3.81 \cdot 10^{-2}$
No. 4	677	$4 \cdot 10^{-2}$	$2.03 \cdot 10^8$	292	$2.4 \cdot 10^{-2}$

where D_n and D_{gamma} are the neutron and gamma dose rates in the channel, respectively, a_1, a_2 and a_3 are constants depending on the neutron and gamma spectrum. The results presented in Table 1 are also plotted on Fig. 1. As these values lie on the direct line defined in the linearity measurements the constants in equation (1) may be united into one single constant. This is in accordance with the assumptions made in the spectrum calculations: the reactor core was idealized as infinite half space, the reflector as a two-dimensional infinite slab. As a result of this approximation the neutron spectrum and the neutron-dose to gamma-dose ratio depend only on the reflector thickness, but not on the core arrangement.

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Effect of Differences in the Elemental Composition of Human Phantoms on the Absorbed Energy Fraction of External Photon Radiation

(Short Communication)

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(Received December 21, 1971)

The elemental composition of the human body depends on several factors such as age, sex, natural environment, etc. However, for phantom measurements and calculations average values characteristic of the given population have to be chosen.

A number of body compositions have been published in past years. Naturally, these are not identical with each other, there are smaller or greater differences between them. The effect of these differences on the absorbed fractions of external photon radiations (i.e. the ratio of the absorbed and incident energies) is the subject of the present study.

Calculations

The well-known Snyder-type phantoms (Fisher, Snyder, 1968; Snyder et al., 1969) were used in calculations by the Monte Carlo code DISDOS (Koblinger, 1971). Compton scattering, photoelectric process and pair-production were taken into account with the most recent cross-section data (Hubbell, 1969).

The calculations were carried out with the following assumptions:

1. The photons are incident in a parallel beam at right angles to the plane determined by the major axis of the trunk ellipse and the vertical axis (see Fig. 1).

2. The total energy of a photon is absorbed at the site of interaction if during a collision it falls below 3 keV or when a photoelectric process occurs.

3. In case of pair-production the kinetic energy of the charged particles is also absorbed locally, and two new photons of energy $m_e c^2$ (0.51 MeV) are emitted.

From the data available in the literature three compositions averaged for the whole body were selected. The first set of figures (Zerby, Kinney, 1965) is very similar to the ICRP standard man (ICRP, 1959), but only the four major elements are considered. The second (Ritts et al., 1970) and the third (Snyder et al., 1969) compositions contain 10 elements (only elements contributing more than 0.1 per cent in weight are taken from the original publications). The composition of the third homogeneous phantom was calculated as the weighted average of values given by Snyder et al. (1969) for an inhomogeneous phantom containing three

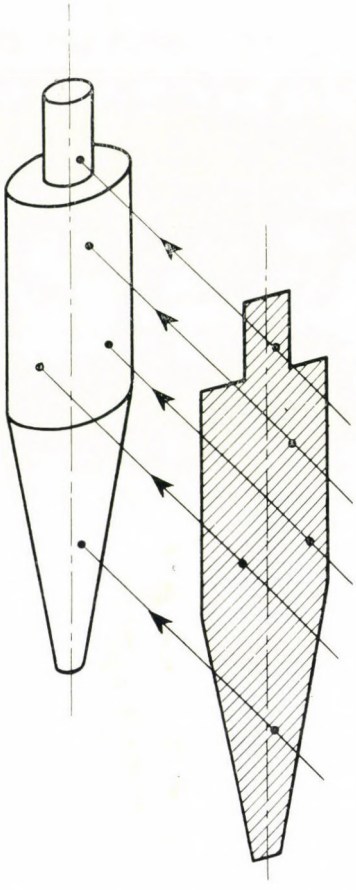

Fig. 1. The shape of the phantom and configuration for the external photon radiation

regions (skeleton, lungs, and remainder of the body) of different compositions and densities.

The inhomogeneous phantom was also included in the study for purpose of comparison. Moreover, calculations were carried out for water to investigate the applicability of water phantoms for measurements.

All compositions are given in Table 1.

Results and conclusions

The absorbed energy fractions for the total body are also presented in Table 1. The standard deviations (characteristic of the statistical error of the Monte Carlo calculation) are less than 1.5 per cent at energies below 0.1 MeV and less than 3.5 per cent in the range from 0.1 to 3 MeV.

Table 1

Absorbed energy fractions in phantoms of different elemental compositions

Tissue	Homogeneous phantoms				Snyder-type inhomogeneous phantom		
	H_2O	Zerby	Ritts	Snyder	Bone	Lungs	Remainder
Elements	Elemental composition (weight %)						
1 H	11.19	10.47	10.00	10.00	7.04	10.21	10.48
6 C	—	18.72	24.00	22.80	22.82	10.00	23.03
7 N	—	3.12	2.90	2.57	3.87	2.80	2.33
8 O	88.81	67.69	60.00	61.30	48.62	76.00	63.23
11 Na	—	—	0.20	0.16	0.32	0.19	0.13
15 P	—	—	1.10	1.20	6.95	0.08	0.24
16 S	—	—	0.24	0.21	0.17	0.23	0.22
17 Cl	—	—	0.20	0.14	0.14	0.27	0.14
19 K	—	—	0.20	0.19	0.15	0.20	0.20
20 Ca	—	—	1.20	1.43	9.92	0.01	—
Density (g/cm^3)	1.0				1.5	0.3	1.0
Energy (MeV)	Absorbed fractions for the total body						
0.01	0.997	0.997	0.998	0.996	0.982		
0.02	0.971	0.969	0.978	0.966	0.878		
0.03	0.886	0.880	0.908	0.856	0.755		
0.05	0.647	0.605	0.695	0.599	0.516		
0.1	0.386	0.382	0.425	0.384	0.357		
0.3	0.364	0.376	0.377	0.372	0.358		
1.0	0.371	0.358	0.361	0.364	0.355		
3.0	0.269	0.280	0.283	0.293	0.262		

The calculated results show that the absorbed fractions are nearly the same for the three homogeneous phantoms and that the water phantom seems to be a very good approximation. The reason for this is as follows: The cross-sections of the different tissues for Compton scattering and pair-production are nearly the same. Greater differences occur only between the photoelectric cross-sections, but this process is dominant at very low energies only, where the free path length is so short — i.e. collisions are so frequent — that nearly all of the energy of a photon is absorbed at the surface of the body, independently of the kind of tissue.

The absorbed fractions for the inhomogeneous phantom are less than those for the homogeneous version, and the differences between the different phantoms having the same average compositions are generally greater than the differences between the four homogeneous phantoms of different compositions. This greater difference is a consequence of the presence of the lung regions.

It is worth noting that the curve of absorbed fraction vs. energy for parallel beam radiation is very similar to that for sources uniformly distributed in a total body (Snyder et al., 1969).

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Continuity of Thick and Thin Filaments

(Preliminary Report)

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Central Laboratory, Medical University, Pécs

(Received January 25, 1972)

One filament models (Hodge, 1955; Ernst, Benedetzky, 1962), two filament models (Huxley, 1953) and three filament models (Guba, 1964; Hoyle, 1968) were described in connection with the longitudinal elements of the fibril. (For literature of this problem see e.g. Ernst, 1970.) Joining this question the present paper reports an observation connected with the filament structure of the fibril.

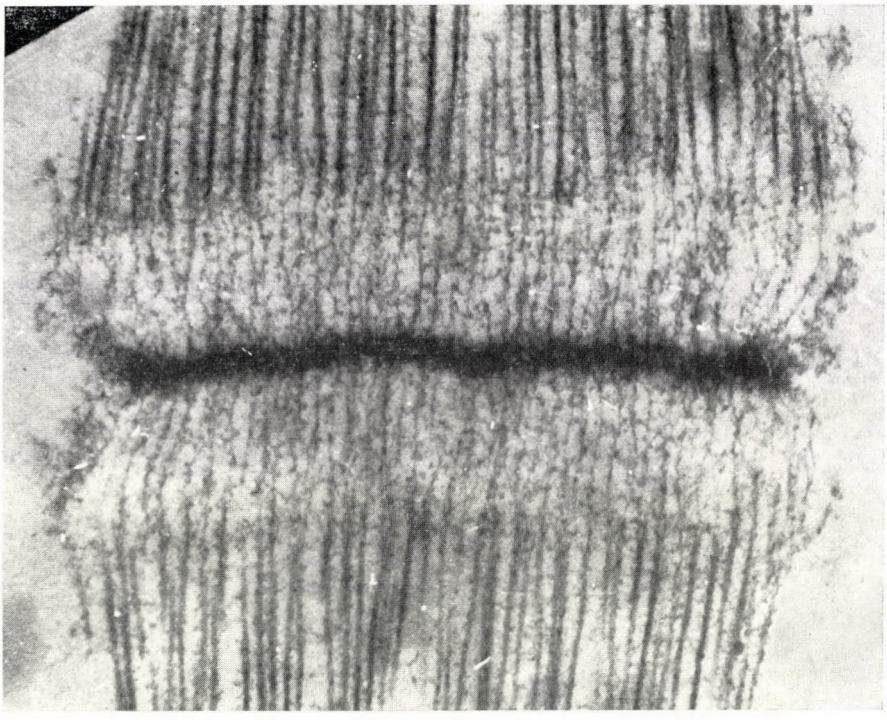

Fig. 1. Fibril (honey-bee) treated in stretched and strained state; the thick filaments of section A continue in the thin filaments of section I. Transversal bridges between the thin filaments. × 60 000

Flight muscle of honey-bee (Apis mellifica) was washed in Pringle solution for 1 to 2 hours, put into 0.07 M sodium-cacodylate buffer of pH 7.4 (containing 0.28 D-glucose; Reanal, Budapest) (where the free muscle contracts spontaneously), then fixed immediately in a 1 per cent osmium-tetroxide solution. After one hour of fixing the muscle was homogenized in a Potter homogenizer for some minutes and then separated in a laboratory centrifuge at some hundreds *g*. The sediment was embedded into araldite and examined in a thin section in the usual way; the supernatant was examined by positive staining after dropping it on a grid with a formvar film without purification.

In the case of stretched and strained muscle fibers a complete lack of overlapping zone was observed in the fibrils (Fig. 1). *The thick filaments of section A continue in the thin filaments of section I and extend as far as Z* (e.g. Garamvölgyi, 1965). Transversal bridges can be found between the filaments of section I.

In the supernatant we can partly observe particles of about 50 Å, partly single chains constituted by these particles, partly double chains constituted by the same particles.

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J. LADIK

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The Heterogeneity of Informofer

I. Different Informofers and Their Role in the Selective Transport of Nuclear dRNA

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(Received February 14, 1972)

By the repeated extraction of cell nuclei at ionic strength (0.3) higher than the usual, further 20–30% of newly synthesized dRNA can be obtained. This dRNA fraction, which mainly comprises dRNA sequences degraded in the nucleus, forms complexes with informofers the protein composition of which is simpler than that of informofers described earlier. This ribonucleoprotein can be found under physiological conditions in the form of polysome-like complexes, but if isolated in the absence of RNase inhibitor it decomposes to 30 *S* particles. The sensitivity towards RNase of particles extracted with buffer containing 0.3 M NaCl is greater and their stability is markedly diminished as compared with 30 *S* particles prepared in the usual way. Their buoyant density in CsCl density gradient decreases from 1.40 gcm^{-3} to 1.37 gcm^{-3} already if the solutions do not contain RNase inhibitor. The heterogeneity of the informofer presumably plays an important role in the intranuclear selective transport of dRNA.

According to the model proposed by Georgiev the transcriptional unit of DNA in eukaryotic cells is similar to the prokaryotic operon, but in the former the acceptor zone is considerably longer, it may constitute about 50% of the operon. The RNA sequences complementary to the acceptor zone of the transcriptional unit can be found at the 5'-end of the newly formed giant dRNA, and is rather rapidly degraded on the effect of intranuclear endo- and exonucleases, whereas the informative part is split into portions corresponding to the structural genes during the course of nuclear transport (Georgiev et al., 1972). The intranuclear transport of dRNA is all the more important, as there is an appreciable dRNA pool in the nucleoplasm. Accordingly, the efflux of information from the nucleus may also occur at the expense of this pool without the simultaneous increase of transcription (Scherrer et al., 1970).

It is known from the data of literature that the dRNA sequences degraded in the nucleus are bound to the chromatin more strongly than is mRNA. In addition, a considerable part of the former can be obtained in the phenolic heat fractionation only if the extraction is performed at higher temperature (85°) (Arion, Georgiev, 1967), or if the ionic strength of the extracting medium is increased (Drews, 1969).

Samarina et al. (1968) have shown that with Tris buffer, pH 8.0, containing 0.1 M NaCl not only ribonucleoproteins containing mRNA can be extracted from the cell nucleus, but dRNA sequences degraded in the nucleus are also

encountered in the extract. On the other hand, Drews (1969) found that if after extraction with Tris buffer, pH 8.0, containing 0.1 M NaCl the nuclear residue was extracted again with the same buffer but containing 0.3 M NaCl, the RNA's found in the two extracts were markedly different. It could be shown on the basis of ^{32}P-nucleotide composition, sedimentation coefficients and hybridization properties that with buffer containing 0.1 M NaCl mainly sequences corresponding to polysomal mRNA were extracted from the nucleus, whereas the extract obtained with 0.3 M NaCl-buffer comprised dRNA which was absent from the polysomes.

In the present experiments we seeked an answer to the question whether the dRNA fraction extracted with Tris buffer containing 0.3 M NaCl is of ribonucleoprotein nature or not, and if it is, whether its protein component is identical with that of the known informofer. We found that the extracted dRNA sequences are complexed with proteins, and these complexes are similar to the well-known informofer, but their protein composition is simpler. In addition the particles extracted with 0.3 M NaCl are heterogeneous and their RNA is more sensitive to ribonuclease than are those found in particles extractable with 0.1 M NaCl.

Materials and methods

White rats of both sexes weighing 150–180 g (strain R Amsterdam) were used. In many experiments the animals were administered 20 μCi ^{14}C-orotate 25–30 minutes prior to death. In some experiments double labeling was applied. In these cases the rats were given $Na_2H^{32}PO_4$ (1.0 mCi per animal) two hours, and 20 μCi of ^{14}C-orotate 20 minutes, before killing.

The pure cell nucleus fraction was prepared by the modified Chauveau method (Samarina et al., 1967).

Nuclei were extracted 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 as described previously (Samarina et al., 1967) with STM II (the same as STM I, but pH 7.8–8.0). The pooled extracts are referred to as "0.1 M extract". Then the nuclear residue was extracted twice with STM III (the same as STM II, but containing 0.3 M NaCl). The extracts were pooled ("0.3 M extract").

RNase inhibitor was prepared from rat liver cytoplasm by Roth's method (Roth, 1958). The inhibitor purified with hydroxylapatite was directly added to the 2.2 M sucrose solution, whereas its solution was dialyzed against the appropriate buffer in the case of extracting solutions. The protein concentration of the inhibitor was 6–8 mg/ml both in sucrose and in the extracting buffers.

The nuclear ribonucleoprotein containing dRNA was purified by gel-filtration (Molnár, Komáromy, 1972). The extract was passed through a Sephadex G-200 column (height : width = 20 : 1; flow rate 3–4 $ml \times cm^{-2} \times hr^{-1}$). The column was washed with STM buffer, pH 7.5, and fractions (20 drops) were collected in an automatic fraction collector. The light absorption and radioactivity of the fractions were measured.

The ultracentrifugation of nuclear ribonucleoproteins in sucrose gradient and in CsCl equilibrium density gradient was carried out as follows: A 15–30% (w/w) linear sucrose gradient was used in STM buffer, pH 7.5, containing 1% formaldehyde (the buffer contained 0.01 M triethanolamine instead of Tris). The samples were centrifuged in the 3×5 ml SW rotor of a Janetzki VAC 60 ultracentrifuge at 38 000 r.p.m. at 4°. Fractions of 3 drops were collected and the absorbancy at 260 nm and radioactivity were measured.

The buoyant density of ribonucleoprotein fixed with formaldehyde was determined as described by Spirin et al. (1965). Gradients mixed of 1.30 and 1.50 $g \times cm^{-3}$ CsCl solutions, also containing the material to be tested, were centrifuged in the 8×10 ml rotor of a Janetzki VAC 60 ultracentrifuge at 42 000 r.p.m. for 18 hours at 10°. Fractions of 5 drops were collected. Density was measured in every fifth fraction of the gradient by means of a refractometer.

Acid-insoluble radioactivity was measured in the following way: Non-labeled ribosomal RNA was added to the fraction, then the material was precipitated with trichloroacetic acid in the cold. The precipitate was washed onto a nitrocellulose filter (Synpor 2) with at least 30 ml of ice-cold 5% trichloroacetic acid and the filter was dried with ethanol. Radioactivity was measured with end-window GM tubes (^{32}P) or in a GAMMA liquid scintillation counter (^{14}C). In the latter case a non-polar scintillator (100 mg POPOP and 4 g PPO in 1000 ml toluene) was used.

For the analysis of particular proteins ribonucleoproteins were obtained from the chromatographically purified material. The preparation was digested with 10 μg/ml RNase in the presence of 6 M urea, the proteins were washed three times with 5% trichloroacetic acid, the precipitate was dissolved in 0.06 M potassium acetate buffer, pH 6.8, containing 6 M urea, dialyzed overnight and then subjected to gel electrophoresis in a cationic discontinuous gel and buffer system containing urea (Molnár et al., 1968; Molnár, 1969). The gels were photographed and also directly evaluated in a densitometer (Juhász, Molnár, 1972).

Results

Extraction of the nuclear ribonucleoproteins containing dRNA with salt solutions of different ionic strengths and purification of the extracts

If the nucleus is extracted in the usual way, about half of the newly formed dRNA remains in the nucleus (Samarina et al., 1967). As shown by the distribution of ^{14}C radioactivity, we observed the same phenomenon. However, in the "0.3 M extract" further 18–25% of the radioactivity can be found and only 20–30% of newly synthesized RNA remains bound in the nucleus (experiments 1 and 2 in Table 1).

Extracts prepared in the presence of RNase inhibitor show a different pattern inasmuch as the distribution of radioactivity is similar to the above described

Table 1

*Distribution of acid-insoluble radioactivity in the nuclear extracts and in the nuclear residue**

No. of experiment	Sample	RNase inhibitor	Radioactivity (^{14}C)	
			imp/min	%
1	extract 1	—	15 700	1.5
	extracts 2,3 ("0.1 M")	—	527 220	49.0
	extracts 4,5 ("0.3 M")	—	296 270	27.5
	Nuclear residue		236 200	22.0
2	extract 1	—	74 848	5.5
	extracts 2,3 ("0.1 M")	—	715 400	52.0
	extracts 4,5 ("0.3 M")	—	268 800	19.5
	Nuclear residue		315 600	23.0
3	extract 1	+	26 000	2.6
	extracts 2,3 ("0.1 M")	+	427 000	43.0
	extracts 4,5 ("0.3 M")	+	138 400	14.0
	Nuclear residue		400 000	40.3
4	extract 1	+	38 500	2.9
	extracts 2,3 ("0.1 M")	+	522 750	38.8
	extracts 4,5 ("0.3 M")	+	234 630	17.4
	Nuclear residue		553 000	41.0

* The animals received 20 μCi of ^{14}C-orotate intraperitoneally 20 min before death

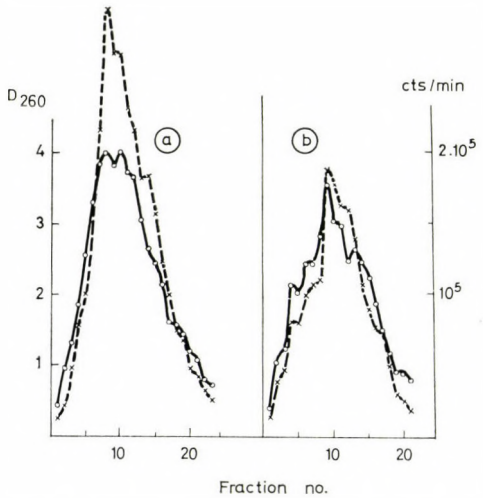

Fig. 1. Elution profiles of "0.1 M extract" (a) and "0.3 M extract" (b) on Sephadex G-200 column. Columns (50×2 cm) were loaded with 5 ml of nuclear extract. After passing through 48 ml buffer, fractions of 20 drops were collected. O—O—O = A_{260}; ×———×———×= radioactivity

but about 40% of the radioactivity originating from ^{14}C-orotate remains in the nucleus after the extractions (experiments 3 and 4 in Table 1).

The "0.1 M extract" and "0.3 M extract" were gel-filtered on a Sephadex G-200 column in order to separate ribonucleoproteins from the contaminating low molecular weight components (Fig. 1). The elution profiles of the two extracts are very similar. In these experiments RNase inhibitor was not used.

Sedimentation behaviour of dRNA-containing ribonucleoprotein extracted with 0.3 M NaCl

It is known that the 30 *S* particles can be regarded as the monomers of more intricate polysome-like complexes (Samarina et al., 1968). On this basis the question should be raised, what are the sedimentation properties of ribonucloproteins extracted with 0.3 M NaCl. If the extraction is carried out in the presence of RNase

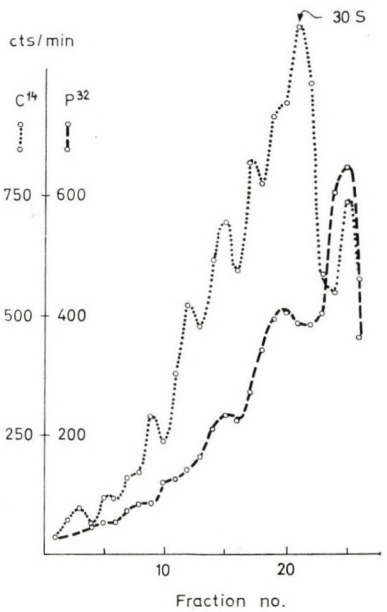

Fig. 2. Sedimentation pattern of "0.3 M extract" prepared from cell nuclei in the presence of RNase inhibitor, in sucrose gradient. The "0.3 M extract" (0.35 ml) was layered on top of 4.8 ml 15—30% (w/w) sucrose in STM buffer, pH 7.5. Centrifugation at 38 000 r.p.m. for 90 min at 4°. Fractions of 3 drops were collected

inhibitor, the "0.3 M extract" is also composed of polysome-like complexes (Fig. 2). If the chromatographically purified materials are centrifuged under identical conditions, the same sedimentation patterns are obtained: a polysome-like profile with less material in the light zone.

If the nuclear extract is prepared without RNase inhibitor, the complexes are decomposed into monomers, the 30 *S* particles, because of the nuclease-induced damage of the long dRNA molecules that connect the informofers (Samarina et al., 1968). In the case of the "0.3 M extract" if no RNase inhibitor is used the

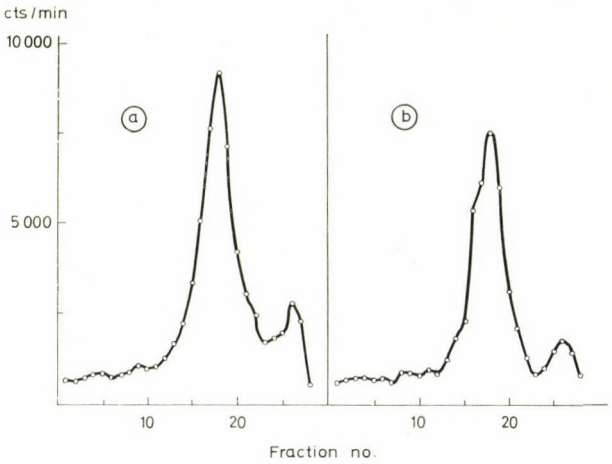

Fig. 3. Sedimentation pattern of "0.1 M particles" (a) and "0.3 M particles" (b) in sucrose gradient after gel-filtration. The filtrate (0.3 ml) was layered on top of 4.8 ml 15–30% (w/w) sucrose in STM buffer, pH 7.5, and centrifuged at 38 000 r.p.m. for 4 hours at 4°. Fractions of 3 drops were collected

decomposition of polysome-like complexes can also be observed and 30 *S* particles are obtained (Fig. 3b). (Radioactive material can be demonstrated in the light zone of the gradient only if the preparation purified by gel-filtration was fixed with formaldehyde prior to gradient centrifugation.)

Proteins of the "0.3 M extract"

Fig. 4 shows the typical electropherograms of the proteins of both "0.1 M particles" (Fig. 4a) and "0.3 M particles" (Fig. 4b). The proteins were isolated from particles purified by gel-filtration. Three major components, A, B and C, were found (Fig. 5), but with "0.3 M particles" the amount of component B markedly increased at the expense of component A, and especially of component C. Before component B, partly in the zone of component A, some material forming a diffuse, non-discrete band can be observed, which is probably composed of proteins released from the chromatin at 0.3 ionic strength. The electropherogram of proteins of the polysome-like "0.3 M particles" is identical with that of "0.3 M" 30 *S* particles.

Fig. 4. Electrophoretic pattern of the proteins of "0.1 M particles" and "0.3 M particles". The tubes were loaded with 130 μg of particle protein, which was previously treated with RNase and washed with trichloroacetic acid. Concentration of polyacrylamide in the gel: 15%. Discontinuous gel and buffer system containing 6 M urea. The inner diameter of glass tubes: 6 mm. Current: 5 mA/tube; running time: 200 min

The heterogeneity of proteins was also examined by SDS polyacrylamide gel electrophoresis. The above differences could also be detected by this method (Molnár, unpublished result).

Stability of particles extracted with 0.3 M NaCl

The previous experiments were performed in the absence of RNase inhibitor. Although even under these conditions particles were abundantly obtained (Fig. 1b), the ultraviolet spectrum shows that in this ribonucleoprotein the protein/nucleic acid ratio is much higher than in the "0.1 M particles" (A_{260}/A_{280} = 1.22 and A_{260}/A_{230} = 0.57 for the "0.3 M particles"). This cannot be the conse-

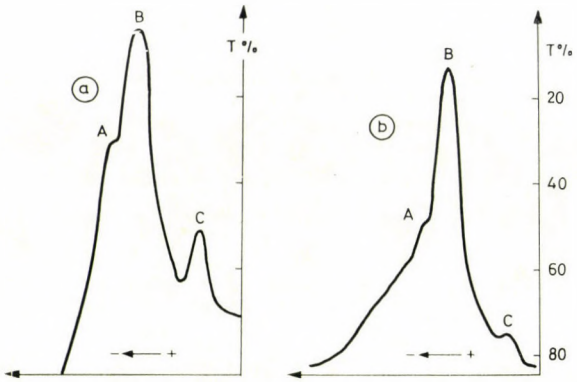

Fig. 5. Densitograms of polyacrylamide gels shown in Fig. 4, recorded in a Zeiss Schnell-photometer

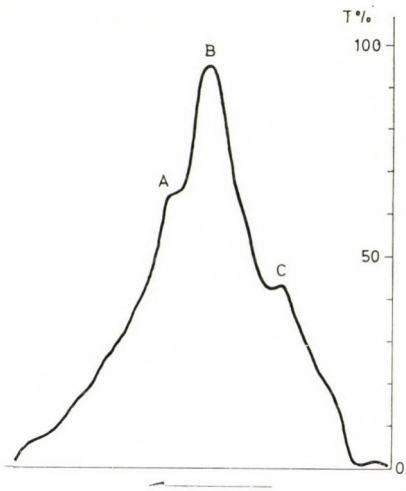

Fig. 6. Electrophoretic pattern of "0.1 M particles" treated with 0.3 M NaCl in polyacrylamide gel. The tubes were loaded with 150 μg of protein. The preparation of samples and the conditions of electrophoresis as in Fig. 4

quence of protein contamination that got into the fraction of particles during gel-filtration, as such an effect could not be detected by polyacrylamide gel electrophoresis. However, the 0.3 ionic strength may cause such an effect by damaging the particles.

If the extraction is carried out in the presence of RNase inhibitor, the ratios $A_{260}/A_{280} = 1.41$ and $A_{260}/A_{230} = 0.54$ are found even for "0.3 M particles", which corresponds to an RNA : protein ratio of 1 : 4 (Samarina et al., 1967).

In an experiment NaCl to 0.3 M final concentration was added to the "0.1 M extract" and allowed to stand in ice cold water for 30 min, then chromatography was

performed in the usual way. The ribonucleoprotein thus obtained did not differ from the control "0.1 M particles" in either the spectrum or protein composition (Fig. 6). It has been shown earlier that the dissociation of particles commences above 0.5 ionic strength and will be complete only at 0.7 M KCl (Samarina et al., 1967a). Accordingly, the diminished RNA content of "0.3 M particles" prepared without RNase inhibitor cannot be due to the elevated ionic strength.

The buoyant density of "0.1 M particles" prepared in the presence of RNase inhibitor was found to be 1.405 gcm^{-3} by centrifuging in CsCl equilibrium density gradient (Fig. 7a). This value is in agreement with our earlier data (Samarina et

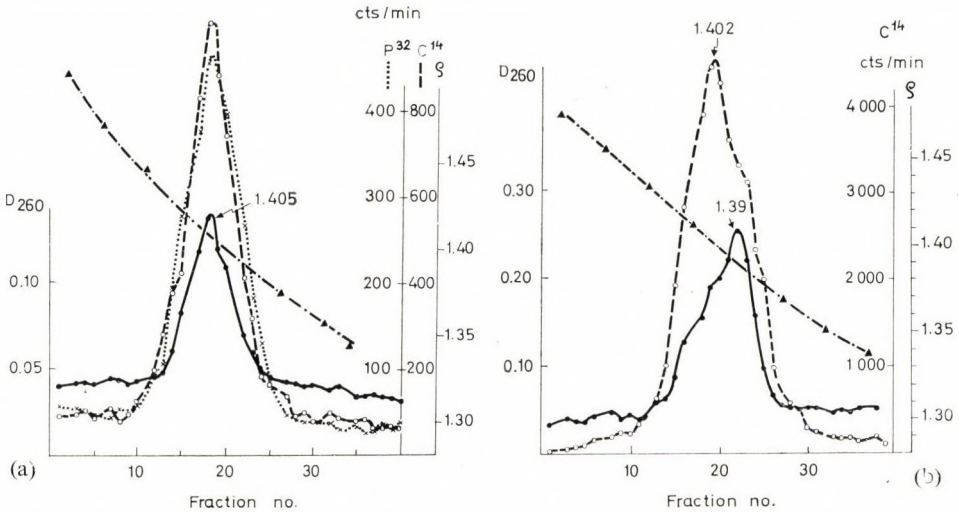

Fig. 7. CsCl density gradient centrifugation of "0.1 M particles" purified by gel-filtration. a = Control. b = Particle preparation, allowed to stand for 5 hours at 0° prior to fixation. $\varrho = 1.30-1.50$ gcm^{-3}, in 0.005 M sodium phosphate buffer containing 2% formaldehyde. Centrifugation was carried out in the 8×10 ml rotor of a Janetzki VAC 60 ultracentrifuge at 42 000 r.p.m. for 18 hours at 10°. Fractions of 5 drops were collected. · · · · ^{32}P radioactivity; ---- ^{14}C radioactivity; —— A_{260}.

al., 1968). If the particles prepared without RNase inhibitor were not fixed just after gel-filtration, but were allowed to stand for 4–5 hours in the cold, the ultraviolet absorption peak did not coincide with the radioactivity peak, and in the latter a definite shoulder could be observed on the light side (Fig. 7b), corresponding to the peak of 1.39 gcm^{-3} density.

The buoyant density of "0.3 M particles" isolated in the presence of RNase inhibitor and purified by chromatography was also found in the expected range, it was 1.40 gcm^{-3} (Fig. 8a). The sedimentation pattern of "0.3 M particles" prepared without RNase inhibitor exhibits unusual features not observed previously (Fig. 8b). The majority of particles are found in zone 1.37 gcm^{-3}, a smaller part in layer of 1.39 gcm^{-3} density. The ultraviolet absorption peak exactly coincides

with the ^{32}P maximum and with the major peak of ^{14}C profile. In the heavier zone (1.39 gcm^{-3}) a definite shoulder can be seen on the ultraviolet and ^{32}P curves, and a separate peak of ^{14}C radioactivity at the same density.

From the changes of buoyant densities and from the above parameters of the spectra of particles the conclusion can be drawn that the "0.3 M particles" lose some of their RNA content during extraction and the subsequent separational manipulations. As the buoyant density of RNA ($\rho = 1.90$) is higher than that of proteins ($\rho = 1.25$) (Perry, Kelley, 1966), the decrease in buoyant density

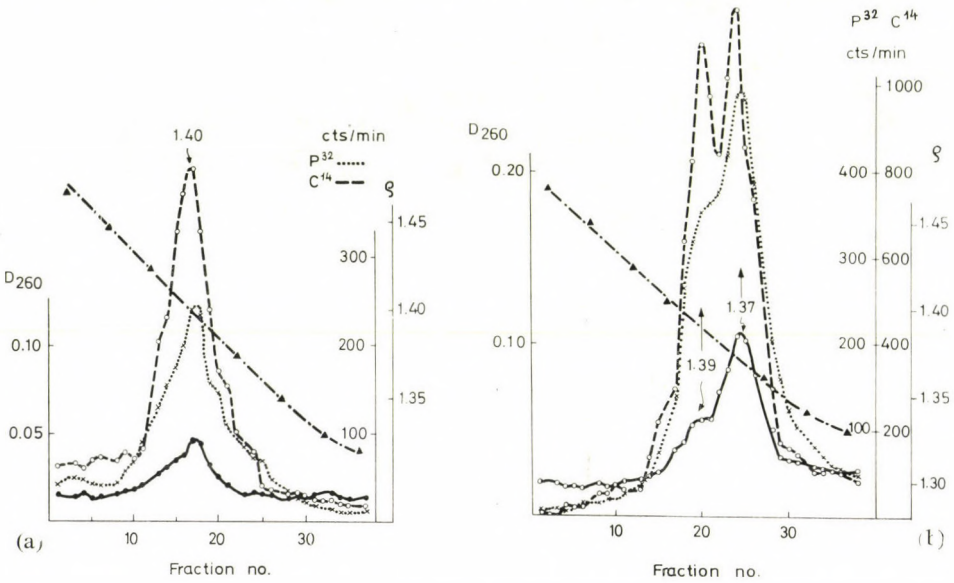

Fig. 8. CsCl density gradient centrifugation of "0.3 M particles" purified by gel-filtration and isolated (a) in the presence and (b) in the absence of RNase inhibitor. The conditions of centrifugation and the notations are the same as in Fig. 7

observed in our experiments can only be interpreted by the decrease of RNA content. The heterogeneity of "0.3 M particles" shown in Fig. 8b can also be observed in electron microscopic pictures (Komáromy, Molnár, in preparation).

Discussion

The ribonucleoprotein nature of "0.3 M particles"

The experiments described above indicate that a further considerable portion of newly formed dRNA can be obtained if the cell nuclei are extracted, after the standard extraction, with higher ionic strength (0.3) buffer. In this way at least about 20% of newly synthesized dRNA can be extracted. This RNA is bound

to informofers and under physiological conditions can be found as polysome-like complexes. This is shown by the finding that if the extracting medium contains RNase inhibitor, polysome-like structures could be extracted. Without RNase inhibitor these complexes are decomposed into 30 *S* particles.

The protein composition of "0.3 M particles" is much simpler than that of "0.1 M particles": component B is predominant, whereas the other two, especially component C, can be found only in small amounts.

The buoyant density of "0.3 M particles" isolated in the presence of RNase inhibitor is 1.40 gcm^{-3} and this value corresponds to a 1 : 4 RNA/protein ratio (Spirin, 1969). If the extracting solution does not contain RNase inhibitor, a part of the RNA of "0.3 M particles" is degraded and dissociated from the particles. For this reason the buoyant density of these particles is 1.37 gcm^{-3}. The elevation of ionic strength to 0.3 does not affect the structure of "0.1 M particles".

The possible role of "0.3 M particles" in the intranuclear transport of dRNA

Only a part of dRNA synthesized in the cell nucleus is transferred to the cytoplasm, where it fulfills the function of mRNA, the rest is degraded in the nucleus (Scherrer et al., 1970; Georgiev et al., 1972). How is this process controlled? It is known that one and the same dRNA molecule contains both mRNA and the part degraded in the nucleus (Mantieva et al., 1971), though contrasting data can also be found in the literature (Penman et al., 1970). In principle, several solutions are possible.

a) The sequence of dRNA itself carries the information on the basis of which the specific nucleases recognize these sequences and hydrolyze the portions to be left behind in the nucleus. This possibility, although suggested by others (Niessing, Sekeris, 1970), does not seem probable in this case as the authors cited could not demonstrate the degradation of dRNA molecules to the acid-soluble stage on the effect of informofer proteins, they could only detect an endonuclease activity which resulted in the formation of 16–18 *S* RNA if 50–80 *S* dRNA was used as substrate.

b) Informofers of different protein composition are complexed with the different types of dRNA. This alternative may allow the formation of a conformational variant of the RNA-protein complex, through one or the other protein component of the informofer, which in one case protects RNA from nuclease attack and is ineffective in the other.

Our present experiments support the view that with dRNA sequences degraded in the nucleus primarily informofers with protein component B are complexed, whereas the other parts of dRNA (mRNA) bind to informofers whose protein moiety is constituted, at least in 50%, by components A and C. The latter would make mRNA resistant to RNase attack.

The cell nucleus fractionation method first applied by Drews (1969) does not permit the isolation of "0.1 M particles" and "0.3 M particles" in high purity. This may be the reason why particles prepared in the usual way contain not only

cytoplasmic mRNA sequences, but also dRNA degraded in the nucleus (Samarina et al., 1968). And if "0.1 M particles" contaminate, even if slightly, the fraction of "0.3 M particles" (which is in fact indicated by the heterogeneity of particles in CsCl equilibrium density gradient), then it is possible that the latter contains only protein component B. This seems to be corroborated by our recent observation according to which in the 30 *S* particles, which are resistant to very high RNase concentrations (5–7 μg/ml), the proportion of protein component C is extremely increased relative to the others (Molnár et al., in preparation).

c) Obviously, the question may be raised: What is the cause of the different protein compositions of the two dRNA types? The dRNA of the nucleus contains DNA (Samarina et al., 1967; Naora, Kodaira, 1968), but the distribution of this among the individual informofer types is not even (Juhász, Molnár, in preparation). Thus in the "0.1 M particles" DNA is presumably present in the form of DNA-RNA hybrids, whereas the "0.3 M particles" are practically void of DNA. This phenomenon may equally influence the different sensitivity towards nucleases and the occurrence of different protein compositions.

The conclusive answer to this question can only be given, when the protein components will be available in pure state and a great variety of complexes can be produced, and studied, with the different types of dRNA or, the other way round, the selectivity of extraction can be improved to such an extent that the resolution of the two types of dRNA becomes feasible.

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Effect of Dietary Fatty Acids on the Desaturation of Stearic Acid in Rat Liver

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Rats were kept on diets containing 10% or 15% tallow or 15% vegetable oil for 2 months, then were injected intravenously with (^{14}C-1) stearic acid. The fatty acid composition of adipose tissue and liver was determined by gas chromatography, and the extent of desaturation of (^{14}C-1) stearic acid in the liver was estimated by thin-layer chromatography.

With animals fed on vegetable oil the lipids of adipose tissue became similar to the food as a result of the decrease in palmitic and palmitoleic acids and the large increase in linoleic acid. On the other hand, with animals fed on tallow the level of stearic acid increased only slightly though tallow contains 30% stearic acid, whereas the level of oleic acid surpassed 50%, i.e. it became higher than in tallow or in the adipose tissue at the beginning. The content of linoleic acid decreased to 3% in animals kept on 15% tallow.

Similar changes were observed with the liver. In addition, with animals kept on tallow arachidonic acid level fell, whereas that of eicosatrienoic acid rose. The latter attained 8% in animals fed on 15% tallow.

Of the (^{14}C-1) stearic acid taken up by the liver, 11%, 4% and 1% were transformed into monoenoic acid with rats fed on 15% tallow, 10% tallow and vegetable oil, respectively. The more stearic acid was in the food, the stronger was desaturation and the more oleic acid was in the tissues.

It has been convincingly shown that the fatty acids of food can alter the composition of fat in animals (Hilditch, 1956). In the simplest case dietary fatty acids are added, in unchanged composition, to the endogenous fatty acids. The situation becomes more complicated if the animal accumulates the individual exogenous fatty acids to different extents, if they are transformed prior to storage in adipose tissue, or if some fatty acids influence the formation of others. In the metabolism of polyenoic fatty acids abundant examples can be found of this last process. It is assumed that the different polyunsaturated fatty acids compete with one another for desaturase, the desaturating and transacetylating processes compete for the fatty acids, and finally the highly unsaturated fatty acids formed during these reactions inhibit desaturase (Brenner et al., 1965; Brenner, Peluffo, 1966, 1969; Nervi et al., 1968; Brenner, 1969).

Of the fatty acids having one unsaturated bond the most important is oleic acid, which is in general the fatty acid found in animal lipids in the largest amount. Fatty acid synthetase does not directly produce unsaturated fatty acids, but Schoenheimer and Rittenberg (1937) have already shown that rats are able

to transform stearic acid labeled with deuterium into oleic acid. Bloomfield and Bloch (1960) studied the desaturation of palmitic and stearic acids into monoenoic acids in yeast extract and found that these fatty acids had to bind to CoA for this process to take place and NADPH and molecular oxygen were also indispensable. Marsh and James (1962) have pointed out that the microsomes isolated from rat liver are able to desaturate stearyl-CoA to oleic acid and here, too, NADPH and O_2 are needed. Uchiyama et al. (1967) studied the effect of fatty acids on the stearic acid desaturating ability of a rat liver preparation in vitro. They found that oleic acid had only a moderate inhibitory effect, whereas linoleic, arachidonic and linolenic acids were more effective.

In the present work we studied the effect of dietary fatty acids on the desaturation of intravenously administered (^{14}C-1) stearic acid in vivo and the changes in the fatty acid composition of liver and fat tissues were determined by gas chromatography.

Material and methods

Male Wistar rats weighing 70–80 g, which had been maintained on standard rat food (LATI), were fed for two months on a special diet containing 10% tallow (4 animals), 15% tallow (4 animals) and 15% vegetable oil (6 animals), respectively. The fatty acid compositions of the diets are shown in Table 1. As a reference, the fatty acid composition of 6 animals receiving standard food was also determined.

Table 1

Fatty acid composition of diets containing vegetable oil and tallow

	14 : 0	14 : 1	16 : 0	16 : 1	17 : 0	18 : 0	18 : 1	18 : 2	18 : 3
Vegetable oil	0.5	—	7.6	1.2	—	5.1	24.8	59.1	1.7
Tallow	3.8	1,4	21.3	6.0	2.2	30.1	31.4	2.3	1.5

The specific activity of (^{14}C-1) stearic acid (Reanal, Budapest) was 1.379 mCi/mmole. The labeled stearic acid was saponified with a small excess of 0.1 N NaOH and then blood plasma pre-heated to 37° was added to the soap solution. The plasma was obtained from animals fed on standard diet. The mixture was shaken vigorously, allowed to stand for a few minutes, then filtered. Until the moment of injection rats were allowed to eat ad libitum. Into the animals tied without anesthesia 0.5 ml blood plasma containing 1 μCi (^{14}C-1) stearic acid was injected. Five minutes after the administration of labeled fatty acid the neck of the rats was broken, their liver and epididymal fat pads were cut out, weighed, quickly rinsed with physiological salt solution and homogenized in chloroform–methanol (2 : 1) mixture.

The homogenates were extracted as described by Folch et al. (1957). Part of the extract was used for the determination of total radioactivity of the tissues.

Another portion of the extract was evaporated in a Rotadest (KUTESZ, Budapest) apparatus in CO_2 atmosphere, taken up in 1 ml hexane and 5 ml HCl–methanol, then the mixture was sealed in a test tube and incubated at 80° for 4 hours in order to transmethylate fatty acids.

From an aliquot of the methylesters the fatty acid composition was determined by gas chromatography. A CHROM III. IKZ (Laboratorni Pristroje, Czechoslovakia) gas chromatograph equipped with a flame ionization detector was used. Column length 2 m, inner diameter 6 mm. The stationary phase was 15% ethyleneglycol succinate adsorbed on 80–100 mesh Chromosorb W. Nitrogen was used as carrier gas, flow rate 100 ml/min, column temperature 184°.

Another aliquot of the methylesters was chromatographed on a 0.33 mm × 20 cm × 20 cm silica gel layer containing 12% $AgNO_3$. Benzene was used as developing solvent. The indicator was Rhodamin B. The bands comprising the saturated, monoenoic and polyenoic fatty acids were separately scraped off and the materials were extracted in a microsoxhlet apparatus for 2 hours with diethyl ether. Finally ether was evaporated from the extracts, and the fat was dissolved in 8 ml of scintillating liquid. The scintillating liquid contained 4% PPO and 0.1% POPOP in toluene. Radioactivity was measured in a USE-2 (Biuro Urzadzen Techniki Jadrovej, Poland) scintillation counter.

Results

In the experiments the external symptoms of essential fatty acid deficiency described by Burr and Burr (1929) could be observed in none of the animals. However, the effect of diet could be noticed in the growth of rats, inasmuch as the growth of animals fed on tallow was retarded as compared with those feeding on vegetable oil. Rats fed with 15% tallow gained weight at the lowest rate.

There were considerable differences in the fatty acid composition of adipose tissue between animals maintained on different diets (Table 2). In animals fed

Table 2

Fatty acid composition of adipose tissue in animals fed on different fats
Mean value ± standard error

	Standard food	10% tallow	15% tallow	15% vegetable oil
14 : 0	3.0±0.3	2.0±0.1	2.3±0.1	1.0±0.1
14 : 1	1.1±0.1	1.0±0.1	1.3±0.1	0.4±0.1
16 : 0	23.7±0.7	22.3±1.0	20.6±0.5	13.2±0.4
16 : 1	10.3±0.6	7.9±0.4	7.7±0.4	3.9±0.1
17 : 0	1.0±0.1	1.2±0.1	1.1±0.1	0.2±0.1
17 : 1	1.2±0.1	1.2±0.2	1.1±0.1	0.4±0.1
18 : 0	4.4±0.2	8.6±0.5	10.8±0.7	4.1±0.1
18 : 1	27.7±0.6	46.0±1.4	50.7±0.9	30.2±0.4
18 : 2	25.7±0.9	8.3±0.6	3.3±0.2	45.3±0.6
18 : 3	1.9±0.4	1.5±0.3	1.1±0.2	1.3±0.1

on vegetable oil linoleic acid level increased to 45%, whereas palmitic acid decreased to 13% and palmitoleic acid to 4%. Thus the lipids of the adipose tissue became similar to dietary lipids. In animals fed on tallow the great fall in linoleic acid level is the most conspicuous, on the effect of diet poor in linoleic acid. In this group the amounts of palmitic and palmitoleic acids hardly changed. Whereas tallow contained 29% stearic acid, even in the adipose tissue of animals fed on 15% tallow for two months only 11% stearic acid was found, but oleic acid amounted to 50% and thus considerably surpassed both its content attained on the original food and the oleic acid level of tallow.

Table 3

Fatty acid composition of liver in animals fed on different fats

Mean value ± standard error

	Standard food	10% tallow	15% tallow	15% vegetable oil
14 : 0	0.8±0.1	0.6±0.1	0.5±0.1	0.3±0.1
14 : 1	0.8±0.1	0.7±0.1	0.6±0.1	0.5±0.1
16 : 0	16.3±1.0	15.1±1.5	15.2±0.8	11.3±0.3
16 : 1	2.8±0.4	3.1±0.2	2.5±0.4	1.6±0.3
17 : 0	1.8±0.4	1.4±0.3	1.7±0.1	1.0±0.2
17 : 1	0.9±0.4	0.4±0.1	0.6±0.1	0.5±0.1
18 : 0	22.4±0.9	18.7±1.7	21.7±0.8	18.0±1.2
18 : 1	11.8±0.4	22.9±2.0	25.6±0.8	12.2±0.7
18 : 2	14.3±0.5	10.5±1.2	4.7±0.6	22.7±0.5
18 : 3	0.9±0.2	0.7±0.2	0.7±0.2	1.5±0.3
18 : 4	0.5±0.1	0.5±0.1	0.5±0.2	0.9±0.1
20 : 3a	0.5±0.1	2.1±0.7	8.6±0.7	0.5±0.1
20 : 3b	1.8±0.1	1.0±0.1	0.9±0.1	0.8±0.4
20 : 4	18.7±1.2	15.3±1.2	9.2±0.5	21.2±0.5
20 : 5	0.7±0.1	0.4±0.2	0.9±0.3	0.5±0.2
22 : 3	0.7±0.1	0.8±0.2	0.7±0.1	1.5±0.3
22 : 4	0.9±0.1	0.8±0.1	0.5±0.4	0.3±0.1
22 : 6	3.4±0.4	5.0±0.1	5.0±0.2	4.7±0.3

20 : 3a = 5,8,11-eicosatrienoic acid
20 : 3b = 8,11,14-eicosatrienoic acid

Similar differences could be revealed in the fatty acid composition of liver (Table 3) between the various groups. In animals fed on vegetable oil, the content of palmitic and palmitoleic acids decreased, stearic and oleic acids did not change, and markedly increased the level of linoleic acid in the liver. In the liver of rats fed on tallow, in spite of the large amounts of stearic acid consumed, the level of stearic acid did not rise, here too oleic acid content increased. With these groups the level of palmitic and palmitoleic acids remained unchanged, whereas the amount of linoleic acid sharply fell also in the liver. Arachidonic acid decreased, too, whereas the otherwise about 0.5% eicosatrienoic acid strongly increased and

in the group fed on more tallow amounted to more than 8% of total fatty acids. Both in adipose tissue and liver the above alterations are more pronounced with animals fed on 15% tallow than with animals that received only 10% tallow.

In animals fed on vegetable oil, 15% tallow and 10% tallow, 12.7 ± 1.2, 10.3 ± 1.3 and 11.4 ± 1.3% respectively, of the labeled stearic acid injected into the femoral vein could be demonstrated in the liver. In the polyenoic fatty acids there was no measurable radioactivity, therefore in Fig. 1 only the activity of monoenoic fatty acids is indicated in percentage of the radioactivity of total fatty acids, the rest of radioactivity belongs to saturated fatty acids. It is seen

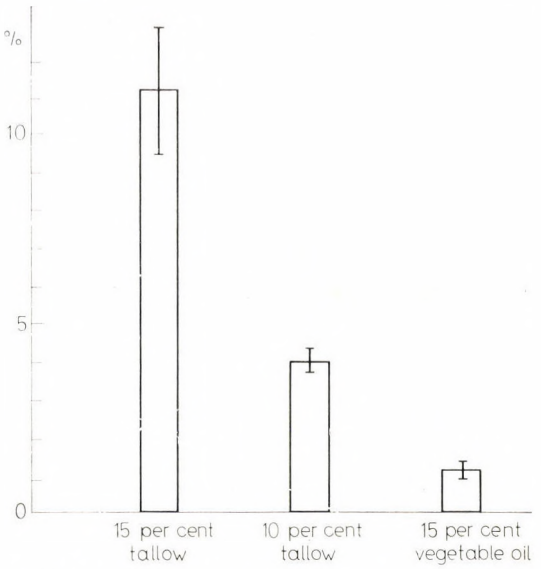

Fig. 1. Effect of dietary fats on the desaturation of (^{14}C-1) stearic acid in the liver. Mean value $\pm$ standard error. The radioactivity of monoenoic fatty acids is expressed in percentage of radioactivity of total fatty acids

in Fig. 1 that stearic acid hardly desaturated in aminals fed on vegetable oil, and there was significant difference ($P < 0.02$) between the two groups fed on tallow, inasmuch as animals fed on 15% tallow desaturated more intensively than did those fed on only 10% tallow.

Discussion

Our results show that saturated and unsaturated fatty acids are deposited in adipose tissue in entirely different ways. The fatty acids of vegetable oil were incorporated into adipose tissue without any appreciable selection or transformation, whereas in animals nourished with saturated fat the level of oleic acid increased instead of stearic acid. The animals are unable to produce linoleic or linolenic acid and though they can introduce an additional double bond in unsatu-

rated fatty acids between the 6th carbon atom from the methyl end and the carboxylic group, the rate of this reaction is negligible as far as the composition of depot fat in mammals is concerned. Thus the only route through which mammals can influence the average saturation, i.e. physical condition, of their fat is the transformation of palmitic acid into palmitoleic acid and of stearic acid into oleic acid. The significance of the state of depot lipids is indicated by the findings according to which the composition of fat in animals depends upon the temperature (Henriques, Hansen, 1901; Fawcett, Lyman, 1954; Farkas, Herodek, 1964; Herodek 1969). According to the data presented in this paper vegetable oil can be readily deposited in fat tissue and it has no ill effects on the animals if their fat becomes oil-like, i.e. quite fluid at body temperature. In contrast, tallow lipids could not be directly deposited, the melting point-raising effect of saturated fatty acids was warded off by vigorous desaturation of stearic acid to oleic acid prior to deposition in the tissues.

In the liver one of the most conspicuous phenomena was the strong increase of eicosatrienoic acid level in animals kept on long-term tallow diet. This fatty acid was first isolated by Nunn and Smedley-McLean (1938) from the liver of rats fed on lipid-free diet and Mead and Slaton (1956) determined the position of double bonds. Thus the compound is 5,8,11-eicosatrienoic acid. As shown by isotopic studies (Fulco, Mead, 1959) this fatty acid is produced from oleic acid through a mechanism similar to the formation of arachidonic acid from linoleic acid (Steinberg et al., 1956). The accumulation of this fatty acid is the first noticeable symptom of the shortage in essential fatty acids (Klein, Johnson, 1954). Mohrhauer and Holman (1963) kept rats on lipid-free diet for 100 days, the diet being complemented with various amounts of linoleic, arachidonic and linolenic acids. If the quantity of linoleic acid surpassed 1% of the calory content of food, the dermatological symptoms of essential fatty acid deficiency failed to develop. Arachidonic acid was effective in one-third of that amount, whereas linolenic acid could not wipe out the symptoms even if added in large amount. The level of eicosatrienoic acid was decreased by all the three essential fatty acids. In our experiments with animals fed on tallow linoleic acid amounted to 2.2% of total fatty acid content, thus it did not reach 1% of the total calories of food even with animals that received 15% tallow. Accordingly, the skin symptoms must have been developed in the long run, but the reserves of the organism were sufficiently large to prevent this during the two months. On the other hand, the 20 : 3 content already increased, especially in animals that received much tallow. These animals also consumed, on the absolute scale, more linoleic acid, thus the appearance of eicosatrienoic acid depends not only on the absolute quantity of essential fatty acids but also on the other fatty acids of the food.

If the three groups kept on different diets are compared in quantitative terms with regard to the stearic acid content of food, the desaturating ability for labeled stearic acid and the oleic acid content of liver and adipose tissue, always the same order is obtained. The more stearic acid was in the food, the more intensive was the desaturation of stearic acid in the liver, and the more oleic acid could be found

in the lipids of liver and adipose tissue. It is readily conceivable that the oleic acid level of the liver increases as a result of the enhanced desaturation of stearic acid. The fatty acids of food reach the adipose tissue through the liver. In addition, fatty acids permanently circulate between adipose tissue and the liver, the liver forms lipoproteids from the free fatty acids released from adipose tissue, and from part of the lipoproteids fatty acids are again taken up by adipose tissue. As a result of this pathway, the fatty acids incorporated from the food into adipose tissue can be desaturated in the liver to a considerable extent. It is possible that the desaturating activity of adipose tissue is affected by the food as in the case of the liver.

It is not known how the stearic acid content of food influences the desaturating activity of the liver. The most plausible explanation would be to attribute the phenomenon to inductive enzyme synthesis. It is possible that not only stearic acid, or not directly the amount of stearic acid, but also the quantity of linoleic acid affects desaturation. As already mentioned, Uchiyama et al. (1967) found that linoleic acid added to a liver preparation in vitro inhibited the desaturation of stearic acid. In fact, in the present in vivo experiments, too, desaturation was the lowest with animals fed on vegetable oil, i.e. with those consuming the most linoleic acid, whereas the difference between the two groups kept on tallow may be related to the circumstance that from animals fed on 15% tallow linoleic acid was depleted to a greater extent than from those receiving 10% tallow. Further investigations are needed to answer these questions.

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Studies on Proteins and Protein Complexes of Muscle by Means of Proteolysis

IX. Digestion of Myosin by Dissolved Papain

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A procedure was elaborated to prepare the total rod, HMM-S-1 and HMM-S-2 fragments of myosin by means of treatment with dissolved papain.*

Digestion of myosin at low ionic strength with papain insolubilized by binding to cellulose or added in the dissolved state takes the same course: HMM-S-1 and total rods are produced first and the latter are transformed on further treatment into LMM and HMM-S-2.

Total rods, LMM, HMM-S-1 and HMM-S-2 obtained by digestion with dissolved papain proved to be identical with the same fragments obtained by treatment with insolubilized enzyme, as regards ethanol resistance, intrinsic viscosity, disc electrophoretic behaviour and the characteristics of the fragments derived from them by tryptic digestion.

Trypsin splits isolated total rods into HMM-S-2 and LMM subfragments: LF-1, LF-2, LF-3; subsequent digestion of total rods with papain, however, yields only HMM-S-2 and LMM. The fragmentation of LMM by papain is different from that effected by trypsin.

Trypsin-soybean trypsin inhibitor complex added to total rod and myosin solutions considerably increases the disc electrophoretic mobility of these proteins.

The results are discussed in their relation to the structure of myosin.

Introduction

The myosin molecule contains globular as well as fibrous parts. It is the globular region by means of which the hydrolysis of ATP is achieved. The rodlike region constituting half of the molecule in weight and about 95% in length is responsible for the formation of filaments at a low ionic strength. As in living muscles myosin functions in the form of supramolecular filaments, a detailed knowledge of the structure of the rodlike region would be important. In studies to this end the proteolytic method has played a very impor tantrole. A segment of

* Abbreviations: TR, total rod; LMM, light meromyosin; HMM, heavy meromyosin; LF-1, LF-2, LF-3, LMM subfragments; HMM-S1, HMM-S2, HMM-S3, HMM subfragments; "U", unidentified helical proteins; ME, β-mercaptoethanol; EDTA, ethylene-diamine tetraacetic acid; TCA, trichloroacetic acid; DFP, diisopropyl-fluorophosphate; PMSF, phenyl-methyl-sulphonyl-fluoride; TLCK, 1-chloro-3-tosylamide-7-amino-2-heptanone HCl; SDS, sodium-dodecyl-sulfate.

the rodlike region (LMM) was successfully isolated from tryptic and chymotryptic digests (Gergely, 1950, 1953; Mihályi, Szent-Györgyi, 1953; Szent-Györgyi, 1953). A stretch of the rodlike region, HMM-S-2, situated outside of LMM was isolated by Lowey et al. (1967) by proteolysis with trypsin bound to an insoluble polymer. The enzymatically active globular region of the myosin molecule, HMM-S-1, had been isolated earlier (Mueller, Perry, 1961; Kominz et al., 1965; Nihei et al., 1968): on digesting myosin with papain bound to cellulose and thus rendered insoluble, the myosin molecule was found to be split at the boundaries of the globular and rodlike sections, and in this way HMM-S-1 was formed. Lowey et al. (1969) reported that on digestion with insoluble papain, in 0.2 M ammonium acetate myosin is split into HMM-S-1 and the total rod, a fragment representing the entire rodlike section of myosin. At the same time the above authors reported that also HMM-S-2 can be isolated from the appropriate papain digest.

By means of tryptic digestions, Bálint et al. (1968) obtained three smaller subfragments from LMM, named LF-1, LF-2 and LF-3 in the order of their increasing electrophoretic mobility.

It was reported by Bálint et al. (1971, 1972) that in the presence of EDTA HMM-S-2 is split off the myosin molecule by digestion with soluble trypsin as well, and that on prolonged tryptic proteolysis HMM-S-2 is transformed into a shorter fragment, named HMM-S-3. It was also established that HMM-S-2 obtained by digestion with soluble trypsin and with papain represents exactly the same molecule.

In our present work it is shown that on treatment of myosin with soluble papain, in the presence of 0.02 M KCl, in the first phase of the digestion the same total rods and HMM-S-1 are obtained as by Lowey's procedure, i.e. by digestion with insoluble papain.

Employing disc electrophoresis for the first time in this field, we succeeded in following the process of the splitting of the total rodlike part of the myosin molecule in the course of digestion by papain, and could also study the subsequent proteolysis of isolated total rods by soluble trypsin and papain.

Simple methods were worked out for the isolation of total rods, HMM-S-1, LMM and HMM-S-2 formed on digestion of myosin by papain.

Experimental procedures

Myosin was prepared according to Portzehl et al. (1950) with modifications as described by Bálint et al. (1968).

Preparation of insoluble papain, isolation of the total rods obtained by digestion with insoluble papain and determination of the activity of insoluble papain were carried out according to Lowey et al. (1969).

Papain used for preparing the insoluble derivative and for certain digestions was a crystallized preparation of Worthington Biochem. Corp., with an activity of 6×10^{-6} M/min/mg enzyme.

Papain applied for other digestions was a lyophilized CalBiochem preparation which was dialysed against 100 vols. of cold ammonium acetate buffer (0.005 M, pH 7) for 48 hours, then lyophilized. The enzyme obtained in this way possessed an activity of 10^{-6} M/min/mg enzyme. With Worthington papain and with CalBiochem papain (purified by dialysis) digestions were carried out with papain: protein ratios of 1 : 120 and 1 : 20, respectively, as a result of which no distinction could be made between the effect of the two papain preparations.

Trypsin was a crystallized preparation of Serva. Before digestion it was incubated in 0.003 *N* HCl for 16 hours at 37°. Trypsin was standardized according to the recommendations of Feb. G.F. Boehringer by the TCTK test. 525 mU of trypsin were used for 1 mg of protein substrate, corresponding to a trypsin: protein ratio of 1 : 120 (w/w).

Determination of protein concentrations was carried out as described by Gornall et al. (1949).

Disc electrophoresis and the photography of the gels were performed according to Bálint et al. (1968). The amount of protein introduced to one gel corresponded to 200–400 μg of undigested protein.

Viscosity was measured in Ubbelohde viscosimeters. Flow time for the solvent (0.5 M KCl + 0.01 M phosphate buffer, pH 7.2) ranged from 70 to 90 sec at 20°. The stock solution was clarified by centrifugation.

Digestions of myosin, isolated total rods and LMM prepared by soluble papain were carried out at 25°, in a medium of the following composition: 0.003 M EDTA + 0.005 M ME + 0.02 M KCl + 0.02 M phosphate buffer (pH 6.5). Protein concentrations ranged from 3 to 12 mg/ml.

Proteolysis with papain was stopped by the addition of freshly dissolved and neutralized iodoacetic acid in a final concentration of 0.001–0.002 M. As described by Lowey et al. (1969), the sulfhydryl groups of myosin are unreactive under similar conditions.

Activation of papain was effected by incubation for 1 hour at 37° in a mixture of the following composition: 0.05 M freshly dissolved and neutralized cysteine + 0.01 M EDTA (neutralized) + 0.033 M phosphate buffer (pH 7.0) + papain in a concentration of 1–5 mg/ml. After activation the enzyme was immediately used for digestions.

ATPase activity was measured in the presence of Ca^{2+} in 2 ml final volumes, for 3 min at 25°, in the following mixture: 0.05 M Tris-HCl buffer (pH 7.5) + + 0.025 M KCl + 0.01 M $CaCl_2$ + 5 mM ATP + 0.3 mg/ml protein. The reaction was terminated by the addition of 2 ml of 10% TCA. 2 ml of the filtrate was used for determination of phosphorus according to Fiske and SubbaRow (1925).

Precipitation of the digested samples by ethanol or acetone was carried out according to Szent-Györgyi et al. (1960).

Results

It was found in the course of our preliminary experiments that LMM, total rods, HMM-S-1 and HMM often remain in the sample gel. On the addition of trypsin–soybean trypsin inhibitor complex (1 : 3, w/w) to these proteins in a complex to protein ratio of 1 : 30 (w/w) their mobilities are considerably increased. This phenomenon, to which we refer as "entering effect", was observed with any combination of every trypsin and soybean inhibitor tested. Trypsin-ovomucoid complex proved to exert an entering effect too. No entering effect was found, however, with soybean trypsin inhibitor alone or with trypsin inactivated by DFP, PMSF or TLCK. On the ground of our experiments we suppose that the process of polymerization of the gel components may play a role in bringing about the effect. This supposition is based on the fact that when the solution of the sample gel containing all the components necessary for polymerization (including riboflavine) was layered on the surface of the spacer gel and protected from light (i.e. was not polymerized), the proteins mentioned did not migrate into the gel, although trypsin–soybean trypsin inhibitor complex was added in this case, too. After a polymerization of 15 min, however, the total amount of the proteins of the same sample entered the gel.

The effect described was not observed in the case of HMM-S-2 and LMM subfragments: the electrophoretic mobility of these fragments was not influenced at all by the presence of trypsin–trypsin inhibitor complex.From the great number of experiments made on the entering effect, Figs 3 and 5 are characteristic examples.

The processes of the digestion of myosin by dissolved and insolubilized papain were compared by employing disc electrophoresis. The entering effect described above necessitates some comments on the interpretation of the gel-pictures. The position of the fragments (TR, LMM, HMM-S-1, HMM) can be anywhere from the border of the sample gel to the one-third of the running gel, depending upon the conditions.

It is clearly shown in Fig. 1a that, in agreement with our earlier investigations (Bálint et al., 1968), undigested myosin is entirely retained in the sample gel, and after ethanol treatment no protein is redissolved (Fig. 1b). As it is known (Szent-Györgyi et al., 1960), after ethanol treatment only proteins with a high helix content and with no globular parts are redissolved; thus the mixture of these proteins can be well separated from myosin, HMM and/or HMM-S-1; hence all the components in Fig. 1b are helical. The comparison of Figs 1a and b reveals that after a short time of digestion by dissolved papain a great quantity of total rods and some HMM-S-2 are formed. As the 6 min digestion mixture treated with ethanol (Fig. 1b) shows, at this time essentially pure total rods are present which may be contaminated only with LMM of a quantity equivalent to HMM-S-2. Thus, at first total rods and (accordingly) HMM-S-1 are formed. Further fragmentation of total rods gives rise to HMM-S-2 and an equivalent amount of LMM (see below).

The process of the digestion of myosin by insolubilized papain according to Lowey et al. (1969) takes essentially the same course as by dissolved papain.

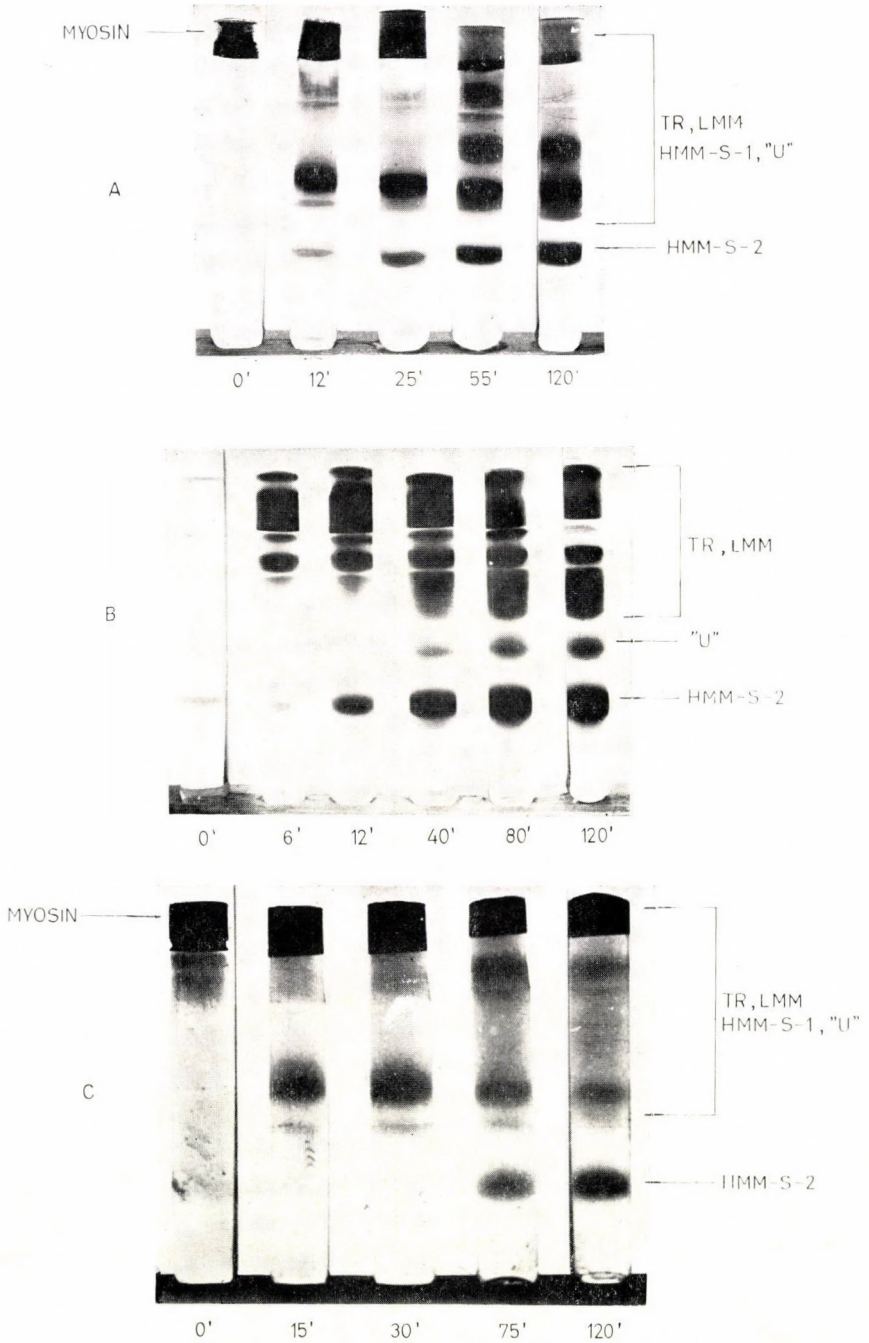

Fig. 1. Papain digestion of myosin. To every gel column samples equivalent to 200 (A and C) and 400 (B) μg of undigested protein were introduced. Digestion times in minutes are indicated under the gel columns. For other details see Methods. For the "U" component see Results. A: total digest, with dissolved papain. B: proteins redissolved after ethanol treatment, digested with dissolved papain. C: total digests with insolubilized papain

The process is considerably slower (compare Figs 1a and c), in spite of the equivalent proteolytic activity present (i.e. 6×10^{-6} M/min/mg enzyme activity added to 120 mg of protein).

Isolation of total rods and HMM-S-1 by limited treatment of myosin with dissolved papain

Myosin was digested at a low ionic strength for 20 min with 6 times less papain than that given in Methods. (This time corresponds to about 3.3 min under the conditions of Fig. 1.) The reaction was terminated by 0.002 M iodoacetic acid, and the digestion mixture was dialysed at 0° overnight against 10 volumes of 0.01 M phosphate buffer (pH 7.0) + 0.001 M iodoacetic acid. The dialysate was centrifuged at $20\,000 \times g$.

The supernatant (HMM-S-1) was lyophilized in the presence of 0.1 M sucrose. For further experiments, lyophilized HMM-S-1 was dialyzed against a solution of 0.02 M KCl + 0.02 M borate-borax buffer (pH 7.6) at 0°.

The precipitate was treated with 3 volumes of ethanol or acetone according to Szent-Györgyi et al. (1960). The suspension in ethanol or acetone was homogenized in a solution containing 0.5 M KCl + 0.01 M phosphate buffer (pH 7.2) and was dialyzed against a solution of the same composition at 0° overnight. After dialysis, the suspension was centrifuged at $6000 \times g$ for 1/2 hour. After this step total rods are in the supernatant fraction.

In this way approximately 140 mg of total rods and 116 mg of HMM-S-1 were obtained from 1000 mg of myosin.

Preparation of LMM, HMM-S-2 and HMM-S-1 by intensive treatment of myosin with papain

In this case myosin was digested for 80 min under the conditions specified in Methods. Termination of the reaction, dialysis and centrifugation were carried out as in the case of the isolation of total rods. Prior to ethanol treatment the LMM precipitate was washed twice with 0.04 M KCl + 0.01 M phosphate buffer (pH 6.5) in order to eliminate contaminating HMM-S-2 (as this fragment is redissolved after ethanol treatment).

The washed precipitate was then treated with ethanol and redissolved by dialysis against a solution containing 0.5 M KCl + 0.01 M phosphate buffer (pH 7.0); the dissolved material was separated by centrifugation. On the ground of intrinsic viscosity measurements, this protein proved to be pure LMM ($[\eta]$ = 1.1 dl/g).

The supernatant of the LMM precipitate containing HMM-S-2 beside HMM-S-1 was immediately treated with ethanol. The redissolved protein proved to be HMM-S-2, contaminated by a small amount of the "U" component (Bálint et al., 1972), a helical fragment soluble at low ionic strength, the relation of which to

the myosin molecule is unknown. This small contamination is always present in the tryptic (Bálint et al., 1972) and papain digests of myosin. Naturally, during this process HMM-S-1 is denatured.

Both HMM-S-1 and HMM-S-2 can be obtained from the supernatant by DEAE cellulose chromatography according to Bálint et al. (1972).

Thus a very simple method for preparing total rods, HMM-S-2 and HMM-S-1 was worked out. The ATPase activity (in the presence of Ca^{2+}, see Methods) of HMM-S-1 obtained in the way described above is 1.67 μM P/mg protein/min after lyophilization, whereas before that it is 2.02.

Characterization of the isolated fragments

The intrinsic viscosity values of HMM-S-1, LMM and total rods prepared as described above and of total rods obtained according to Lowey et al. (1969) are shown in Fig. 2.

Fig. 3 shows the disc electrophoretic picture and the subsequent tryptic digestion of HMM-S-1 prepared by limited digestion with papain. It can be well seen that, in accordance with the observed entering effect (see above), part of the protein introduced remains in the starter gel without trypsin-trypsin inhibitor complex, whereas in the presence of a mixture of trypsin and inhibitor HMM-S-1 gives a homogeneous disc in the running gel. On tryptic digestion this is transformed into an electrophoretically distinct component of greater mobility, as it was observed earlier too (Bálint et al., 1972). This change cannot be due to the degradation of contaminating HMM present as during the process no HMM-S-2 is formed. The enzymatic and molecular characterization of the two kinds of HMM-S-1 will be the subject of another communication.

In Figs 4 and 5 the subsequent digestion by trypsin and papain of total rods* prepared by means of treatment with soluble papain at a low ionic strength is shown.

The subsequent digestion by trypsin and papain of total rods prepared according to Lowey et al. (1969) gave essentially similar results as can be seen in Figs 4 and 5.

In the case of subsequent digestion by papain LMM is degraded very slowly, in a way different from tryptic digestion (Fig. 6).

From these results the following conclusions can be drawn:

1. The two kinds of total rods are split in exactly the same way, whether trypsin or papain is used.

2. HMM-S-2 is formed in the case of both proteins with both enzymes.

3. In the case of tryptic digestions LMM subfragments and HMM-S-3 also appear from both proteins.

* In the experiments represented in Figs 4 and 5 total rods were prepared by acetone treatment.

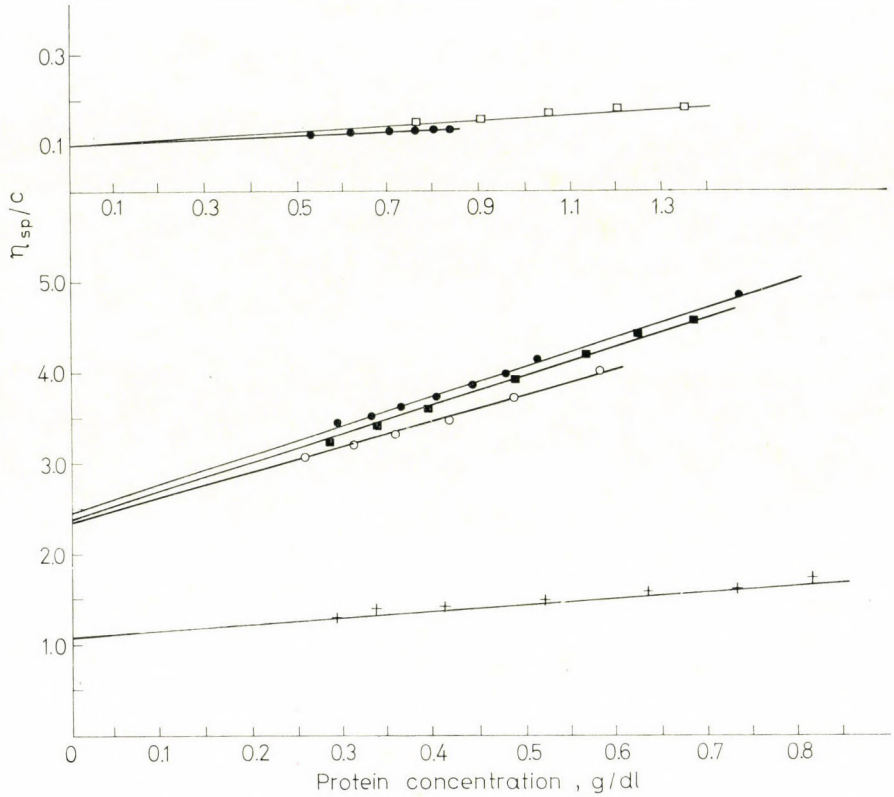

Fig. 2. Concentration dependence of the reduced viscosities of total rods, HMM-S-1, and of LMM obtained by intensive treatment with papain. Upper part: HMM-S-1 (two different preparations, ● and □); lower part: total rods prepared by dissolved papain (two preparations, ○ and ●); total rods prepared by insolubilized papain (■); LMM (+). (For the concentration dependence of the reduced viscosity of HMM-S-1 prepared by intensive papain digestion see Bálint et al. 1971)

4. A proper way of deciding whether a preparation consists of total rods is to test whether on digestion with trypsin or papain HMM-S-2 is formed from it or not (see Figs 4, 5 and 6).

Discussion

The experiments described in this paper demonstrate that splitting of myosin to total rods plus HMM-S-1 can be achieved by treatment of a myosin suspension (at low ionic strength) with dissolved papain. Total rods can thus be prepared without recourse to papain insolubilized by attachment to cellulose as used in Lowey's procedure (Lowey et al., 1969).

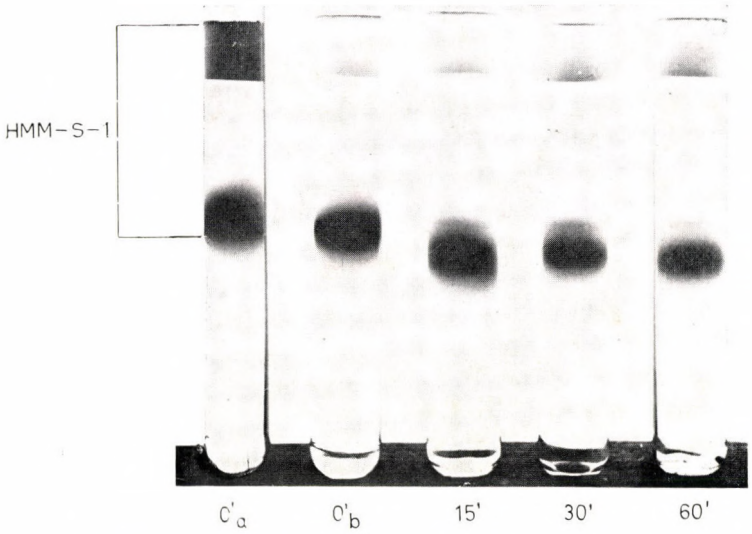

Fig. 3. Subsequent tryptic digestion of HMM-S-1 prepared by short-time digestion of myosin by papain. $0'_a$: the separated and lyophilized substance after redissolving. $0'_b$: the same substance, treated before electrophoresis with trypsin inactivated by 3 weights of soybean trypsin inhibitor, added in a trypsin : protein ratio of 1 : 120. Digestion times are indicated under the gel columns

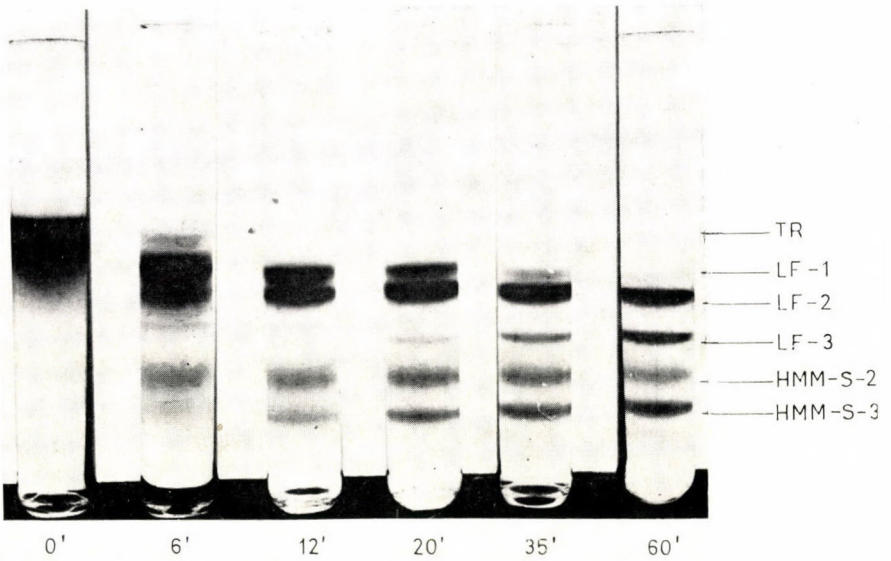

Fig. 4. Subsequent tryptic digestion of total rods prepared by digestion with dissolved papain. Digestion times are indicated under the gel columns. The gel column marked 0' contains a sample to which first trypsin inhibitor and only then trypsin were added

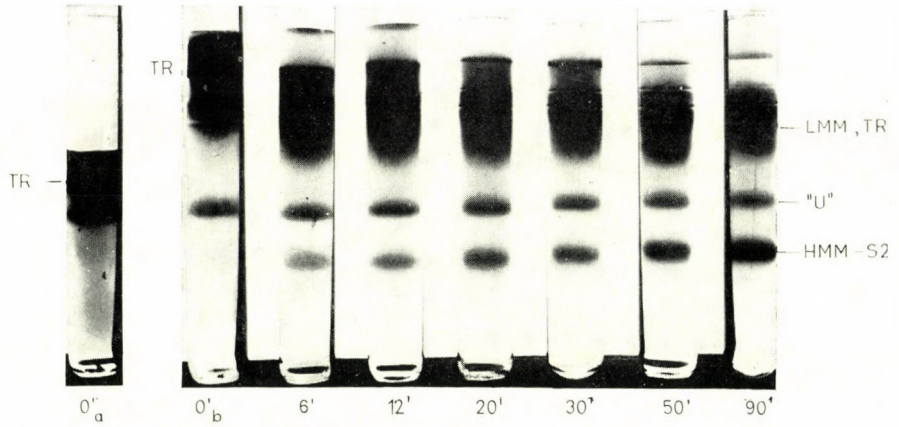

Fig. 5. Subsequent digestion with papain of total rods prepared with dissolved papain. Under the gel columns digestion times are indicated. Gels marked 0' contain proteins to which iodoacetic acid was added first, and then papain. $0'_a$: the sample was treated with trypsin–trypsin inhibitor complex, in a trypsin: protein ratio of 1 : 120. Trypsin was previously inactivated by soybean inhibitor, added in a ratio of 1 : 3. $0'_b$ and other samples were not treated with trypsin–trypsin inhibitor complex

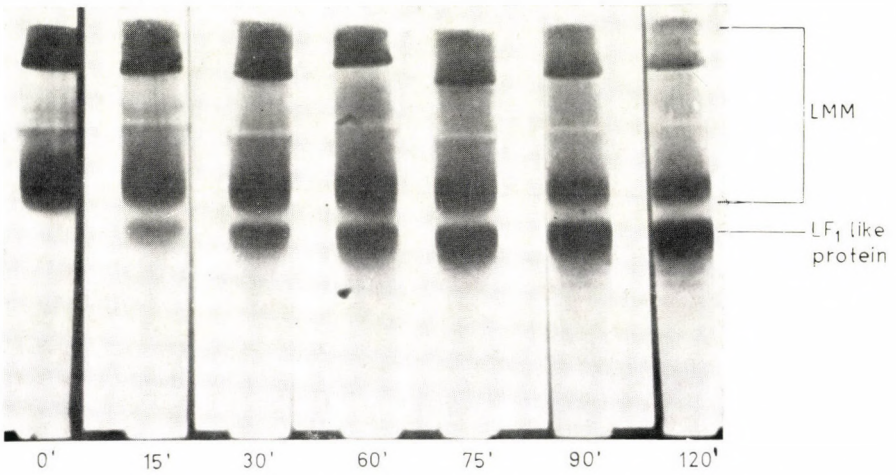

Fig. 6. Subsequent digestion of LMM by papain. — LMM obtained by tryptic digestion (Szent-Györgyi et al., 1960) was digested by dissolved papain as described in Methods. Digestion times are indicated under the gel columns

The finding that on subsequent digestion of total rods by trypsin all the helical fragments observed to arise on tryptic digestion of LMM and HMM – and only these – are formed (Fig. 4), makes the assumption very probable that the

total rod contains no substantial segment absent from LMM plus HMM. This view can be supported by considerations of molecular weights too. The molecular weights suggested by us for HMM-S-2 (72 000; Bálint et al., 1972) and for LMM (144 000; Bálint et al., 1968) add up to 216 000. This value is in excellent accord with the value (220 000 ± 10%) reported earlier by Lowey et al. (1969), as well as with the more recent value (200 000) of Lowey's group (Harrison et al., 1971) based on disc electrophoresis in SDS. Considering the molecular weight accepted for HMM-S-1 (about 120 000 each), one can build up the whole myosin molecule from total rod (or LMM + HMM-S-2) and HMM-S-1's. No allowance is needed for substantial peptide material formed at either primary splitting, i.e. at the splitting of myosin to LMM and HMM by trypsin or to total rods and HMM-S-1's by papain. Although the still prevailing uncertainty as to the right molecular weight of myosin (see Gazith et al., 1970) made this kind of calculations rather moot, the tendency is still towards a decrease of molecular weight, rendering the accommodation of any segments other than the fragments considered here impossible. This conclusion is in accord with similar reasonings of the Lowey group. The "primary splittings" differ thus considerably from the degradation of LMM to its subfragments or of HMM-S-2 to HMM-S-3, as in these processes consecutive segments of some 30 000 daltons are split to peptides.

Concerning this latter process, it is interesting to point out the similarity of the digestion of LMM by papain to that effected by chymotrypsin, as contrasted to the digestion by trypsin. With chymotrypsin (Bálint et al., 1968) or with papain, LMM is first split to a not very homogeneous product approximating LF-1 but on further digestion only peptide material is formed. It may well be that this continuous degradation, in contrast to the effect of trypsin where degradation proceeds in distinct consecutive steps, is due to the relatively high specificity of the latter enzyme.

Some comment is needed on the change of the electrophoretic mobility of HMM-S-1 on prolonged papain treatment. In all probability the change reflects the formation of a smaller molecule from the "short-term" HMM-S-1. The molecular weight and the enzymatic characteristics of this smaller HMM-S-1 have not been studied as yet. It should be pointed out, however, that several HMM-S-1-like fragments have been described which are somewhat smaller than HMM-S-1 obtained by tryptic or papain digestion (Hotta, Usami, 1967; Hotta, Fujiba, 1967; Yagi et al., 1967 a, b).

We described the "entering effect" (see Methods) only because of its rather disturbing methodical implications. We were unable to elucidate its mechanism. From the point of view of the interpretation of disc electrophoretic pictures it is important to stress, however, that in all probability this effect is not caused by residual proteolytic activity. The most convincing experiment against this possibility (among others not described here) is the following observation: if with complete gels (containing all the components) the photopolymerization is delayed by 24 hours, we obtained exactly the same disc pattern as in the case of processing the sample immediately after mixing. We are rather inclined to assume that tryp-

sin–trypsin inhibitor complex (which is present in the same *molar* concentration as the protein components of the sample) has some kind of de-aggregating effect. The elucidation of this phenomenon would of course need further investigations.

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The Effect of Tyrosinase on Glyceraldehyde-3-phosphate Dehydrogenase and Glycerophosphate Dehydrogenase

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Glyceraldehyde-3-phosphate dehydrogenase and α-glycerophosphate dehydrogenase proved to be substrates of tyrosinase. However, GAPD holoenzyme is a rather poor substrate as compared with the apoenzyme.

In the case of glyceraldehyde-3-phosphate dehydrogenase apoenzyme 2 tyrosyl groups per subunit are oxidized in the first hour of incubation with tyrosinase. This is followed by the slow inactivation of the enzyme. The inactivation is probably the result of the oxidation of SH-groups, performed by an oxidation product of tyrosine, identified with thin-layer chromatography. The inactivated enzyme is still able to bind 4 moles of NAD.

The authors have shown that tyrosinase is able to oxidize 3–4 tyrosyl groups of α-glycerophosphate dehydrogenase. However, this oxidation does not alter the enzymic activity.

Introduction

Tyrosinase is a specific, oxidative modifier of tyrosyl side chains of proteins. DOPA and DOPAchrom are the oxidation products in proteins, whereas in the case of free tyrosine the reaction goes further to the formation of melanin (Lerner, 1953).

Cory and Frieden (1967b) investigated the effect of tyrosinase on yeast ADH, rabbit muscle LDH, aldolase, and GAPD holoenzyme. They found the first three enzymes to be, but rabbit muscle GAPD not to be, the substrate of tyrosinase.

We investigated the susceptibility of tyrosyl groups of pig muscle GAPD and rabbit muscle GPD to tyrosinase and the effect of tyrosinase on the function of these enzymes.

Abbreviations:

GAPD = glyceraldehyde-3-phosphate dehydrogenase
GPD = α-glycerophosphate dehydrogenase
α-GP = α-glycerophosphate
DTNB = 5,5'-dithiobis-(2-nitrobenzoic acid)
ADH = alcohol dehydrogenase
LDH = lactate dehydrogenase
DOPA = dioxyphenylalanine

Materials and methods

Four times recrystallized pig muscle glyceraldehyde-3-phosphate dehydrogenase (Előd, Szörényi, 1956) and rabbit muscle glycerophosphate dehydrogenase (Telegdi, 1964) were used. Tyrosinase was prepared from fresh common mushrooms (Psalliota campestris) by the method of Frieden and Ottesen (1959) as modified by Cory and Frieden (1967a).

Glyceraldehyde-3-phosphate was prepared from fructose 1,6-diphosphate, a Reanal product (Szewczuk et al., 1961). NAD was Reanal, α-GP and DTNB were Fluka and tyrosine and DOPA were Calbiochem products.

Nitrosonaphtol was prepared from β-naphtol with sodium nitrite. Urea was used after recrystallization from ethanol. The other chemicals were commercial preparations of reagent grade.

Enzymic activity of GAPD and GPD was determined by the Warburg optical test (Warburg, Christian, 1939). The reaction mixtures: GAPD activity: 1.7×10^{-3} M NAD; 1.9×10^{-3} M GAP; 10^{-3} M arsenate; 7×10^{-9} M enzyme; in 0.1 M glycine buffer, pH 8.5; GPD activity: 4×10^{-9} M enzyme; 1.7×10^{-3} M NAD, 6.3×10^{-2} M GP; in 0.1 M glycine buffer, pH 10.

Enzymic activity of tyrosinase was measured with DOPA substrate, and followed at 475 nm (Fling et al., 1963).

Specific activities: GAPD 15 000 – 20 000 moles of NADH produced × $\text{min}^{-1} \times$ mole of enzyme^{-1}; GPD 25 000 – 30 000 Bücher units (Beisenherz, 1955); tyrosinase 440 tyrosinase units/ml (Fling et al., 1963).

Treatment of GAPD and GPD with tyrosinase: Tyrosinase (50 units/ml) was added at room temperature to $3.6 - 3.9 \times 10^{-5}$ M enzyme in the presence of 10^{-6} M Cu^{2+} ions in 0.1 M Tris buffer, pH 7.5. Enzyme samples treated in a similar manner in the absence of tyrosinase served as controls.

Charcoal treatment for the removal of bound nucleotides of GAPD and GPD resulted in an increase of the A_{280}/A_{260} ratio from 1.05 to 1.95 and from 1.0 to 1.8, respectively.

Protein concentrations were measured on the basis of absorbancy at 280 nm, by using the following extinction coefficients:

GAPD holoenzyme and GPD: $A\,{}^{1\,\%}_{1\,\text{cm}} = 10.0$

Charcoal-treated GAPD and GPD: $A\,{}^{1\,\%}_{1\,\text{cm}} = 8.9$

Tyrosinase: $A\,{}^{1\,\text{cm}}_{1\,\%} = 24.9$

Formation of the charge-transfer complex of GAPD with NAD was measured according to Racker and Krimsky (1952). Tyrosine content of the enzymes was determined with nitrosonaphtol according to Uehara et al. (1970). DOPA does not give this reaction. The error was ±5%.

Determination of SH-groups was carried out by Ellman's method (Ellman, 1959). Thin-layer ion-exchange chromatography (on resin coated chromatoplates Ionex-25 SA, Macherey-Nagel et Co. Düren) of the oxidation product of tyrosine was performed after hydrolysis with 6 N HCl at 105° for 72 hours according to Dévényi (1970).

Results and discussion

The effect of tyrosinase on GAPD apoenzyme and holoenzyme

Solutions of apoenzyme and holoenzyme of GAPD (3.6×10^{-5} M) were incubated with tyrosinase in the presence of Cu^{2+} ions to increase the rate of tyrosinase action (Lerner, 1953). Cu^{2+} ions inhibit GAPD by forming a complex with the reactive SH-group of the enzyme (Cys 149) (Cseke, Boross, 1972). The Cu concentration used in our experiments was 30 times less than that causing inhibition of the enzyme.

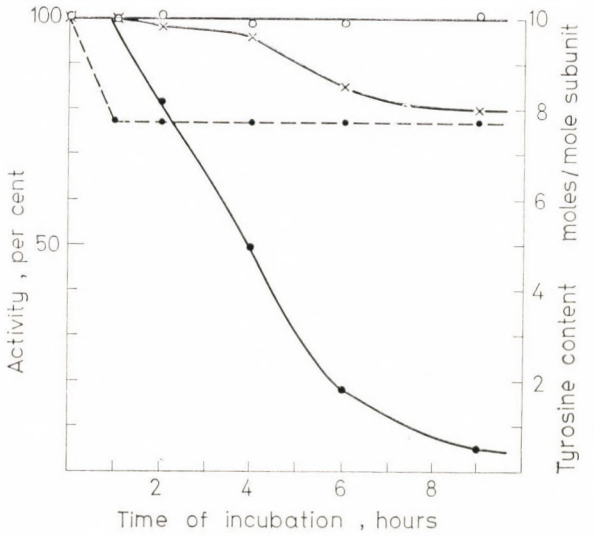

Fig. 1. Inactivation of GAPD apoenzyme and holoenzyme by tyrosinase. Incubation mixture: 3.6×10^{-5} M GAPD apoenzyme and holoenzyme, respectively, 50 units/ml tyrosinase, 10^{-6} M $CuSO_4$, in 0.1 M Tris buffer, pH 7.5, room temperature. The tyrosinase-free controls were kept under the same conditions. Enzymic activity was measured with: 7×10^{-9} M enzyme, 1.7×10^{-3}M NAD, 1.9×10^{-3} M GAP, 10^{-3} M arsenate, in 0.1 M glycine buffer pH 8.5. O—O GAPD control; ×—× GAPD holoenzyme; ·—· GAPD apoenzyme; — — — tyrosine content of GAPD apoenzyme

The effect of tyrosinase was followed by measuring the activity of GAPD.

Nine hours of incubation with tyrosinase at pH 7.5 at room temperature caused 20% decrease in the activity of GAPD holoenzyme, while the control remained fully active (Fig. 1). Tyrosinase, as checked with DOPA, was also fully active at the end of incubation. These results show that GAPD holoenzyme is a poor substrate for tyrosinase.

In the case of GAPD apoenzyme 9 hours of incubation with 50 units/ml of tyrosinase decreased GAPD activity to zero (Fig. 1).

We measured the tyrosine content of GAPD simultaneously. In GAPD apoenzyme during the first hour of incubation 2 tyrosyl groups per subunit were oxidized by tyrosinase and no further decrease in the tyrosine content was detectable afterwards (Fig. 1). It should be noted that although the oxidation of tyrosyl groups takes place in the first hour of incubation, there is no change in the GAPD activity during this time.

The number of tyrosyl groups of GAPD holoenzyme did not change even after 24 hours of incubation. DOPAchrom, the oxidation product of tyrosyl side chains, is able to oxidize the SH-groups of proteins (Lerner, 1953). During treatment with tyrosinase, after the oxidation of tyrosyl side chains, GAPD apoenzyme lost 3 to 4 SH-groups (see Fig. 1, Table 1). This shows that inactivation is caused by the oxidation of SH-groups.

Table 1

Effect of tyrosinase on the SH content of GAPD apoenzyme

Tyrosinase treatment as in Fig. 1. The SH content was measured according to the method of Ellman (1959) in the following mixture: 1.1×10^{-8} M enzyme, 0.1 ml 0.1 M EDTA, 2.5 ml 10 M urea, 0.1 ml 3.9 mg/ml DTNB

Time of incubation, hours	Tyrosinase-treated enzyme	Control
	mole SH/mole enzyme	mole SH/mole enzyme
0	16.1	16.6
1	15.3	15.9
3	14.8	15.9
5.5	12.6	15.9

Inactivated GAPD apoenzyme was incubated for 15 min with 0.01 M 2-mercaptoethanol but no activity could be recovered. This means that either the oxidation of SH-groups was followed by a secondary structural change of the protein molecule which could not be reverted, or the oxidation does not stop at disulfide formation. The holoenzyme could be fully reactivated with 2-mercaptoethanol.

The oxidation product of tyrosine and the decrease in the number of tyrosyl groups could be demonstrated with thin-layer chromatography (Fig. 2). It was found that during the hydrolysis of the protein samples (in 6 *N* HCl at 105°), the oxidation product of tyrosyl side chains, DOPA, decomposed. The decomposition products gave two new spots with Rf values different from that of DOPA. One of these two spots was found in the tyrosinase-treated GAPD apoenzyme, too.

We measured the spectrum of the tyrosinase-treated and control GAPD before and after addition of NAD, between 350 and 380 nm (Racker-band, characteristic of the enzyme-NAD complex). The completely inactivated GAPD

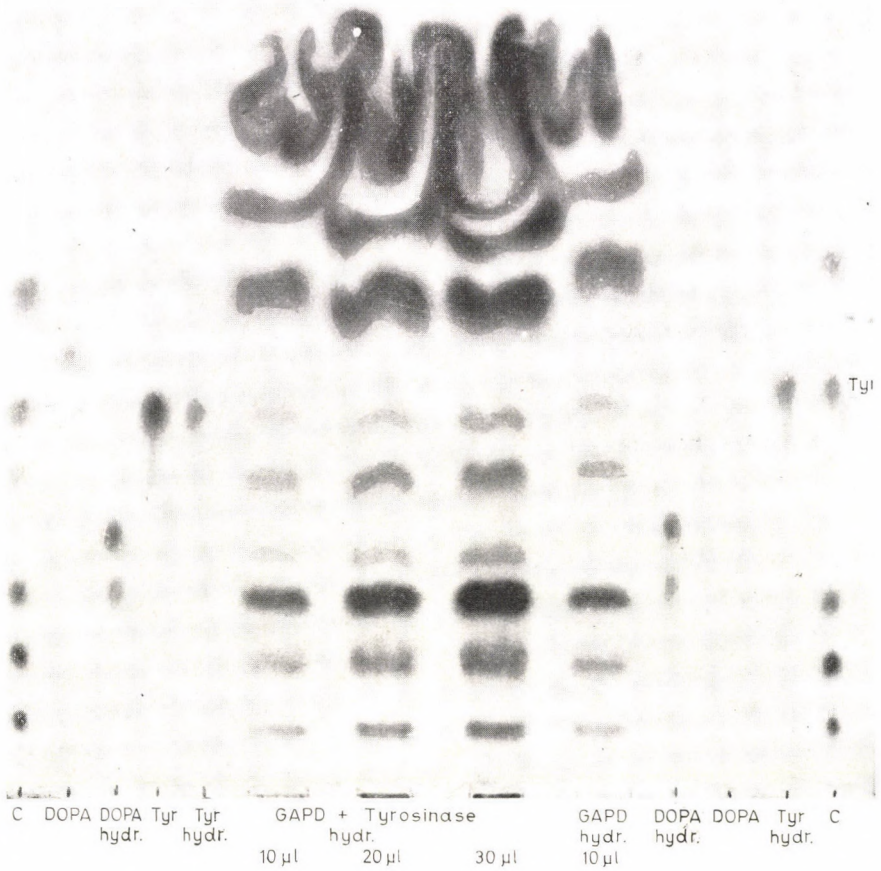

Fig. 2. Thin-layer chromatogram of tyrosinase-treated GAPD apoenzyme hydrolyzate. Tyrosinase treatment as in Fig.1. The protein samples were hydrolyzed in 6 *N* HCl at 105° for 72 hours. The plate was equilibrated for 24 hours in 0.04 *N* Na-citrate buffer, pH 3.28. The run was made at pH 5.28 in 0.35 *N* Na-citrate buffer and the plate was developed with ninhydrin-Cd reagent. C = control mixture of amino acids

apoenzyme is able to bind 3.8 ± 0.2 moles of NAD similarly to the fully active control.

It is concluded that the total loss of enzymic activity of tyrosinase-treated apoenzyme is only indirectly related to the oxidation of two tyrosyl side chains per subunit, these tyrosines are not essential for the enzymic activity. It was shown by stochastic analysis of SH blocking of GAPD that two cysteinyl side chains per subunit participate in the enzyme function (Friedrich, 1967). One of these, Cys 149, forms the charge transfer complex with NAD, the other has not been identified yet. Since the charge transfer complex with NAD still develops after

inactivation by tyrosinase, it is possible that the unidentified essential SH-group is oxidized. The sterically fixed oxidized tyrosine is possibly in the neighbourhood of this SH-group.

Effect of tyrosinase on GPD

The effect of 38 units/ml of tyrosinase on nucleotide containing and nucleotide free GPD was measured. In both cases the tyrosinase-treated samples remained 100% active, 22 hours of incubation did not cause any change in the activity.

We determined simultaneously the tyrosine content of GPD during the incubation with tyrosinase. The data in Table 2 show that GPD is a substrate

Table 2

Effect of tyrosinase on the tyrosine content of GPD

Incubation mixture: 3.85×10^{-5} M GPD, 38 units/ml tyrosinase, 10^{-6} M $CuSO_4$, in 0.1 M Tris buffer pH 7.5. The mixture was incubated at room temperature. Tyrosine content was determined as in the case of GAPD. 2.9×10^{-8} moles of GPD were used (in 1 ml volume)

Incubation time	0	5 min	2h	6h	72h
Tyrosinase-treated GPD	9.6	8.8	8.8	5.3	5.3
GPD control	9.6	9.6	9.6	9.6	9.6

for tyrosinase, since 6 hours of incubation with tyrosinase caused the oxidation of 3 to 4 tyrosyl side chains. Oxidation of SH-groups could not be detected.

We can conclude that although GPD is a substrate for tyrosinase, the tyrosyl side chains, susceptible to tyrosinase, are not essential for the enzyme function. They are not needed even for the maintenance of the native structure of the catalytic site.

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Thin-Layer Ion-Exchange Chromatography on Resin-Coated Chromatoplates

VII. Rapid Determination of C-terminal Sequences on the Nanomole Scale (Short Communication)

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(Received November 16, 1971)

Dedicated to Professor A. E. Braunstein in commemoration of his 70th birthday

The best way to determine the C-terminal amino acid sequence is digestion with carboxypeptidase, during the course of which the amino acids successively liberated from the C-terminus of the peptide or protein can be identified (cf. Ambler, 1967). Amino acids are usually identified by paper chromatography, paper electrophoresis or automatic amino acid analysis.

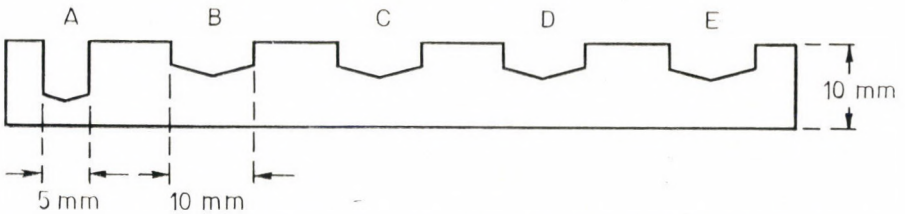

Fig. 1. Multi-hole plexiglass rack for micro-scale digestion. A: tray for digestion; B–E: sampling holes

Paper chromatography or paper electrophoresis gives unequivocal results only in two-dimensional separations. Identification by means of the amino acid analyzer is unambiguous, but the analysis of four or five samples taken at different times lasts for 15 to 18 hours even if the rapid procedures (Dévényi, 1969) are applied.

Among the methods used so far for the analysis of carboxypeptidase digests the amino acid analyzer technique is the most sensitive. By using long-path cuvettes, at a sensitivity of 5 nmoles, about 20 to 30 nmoles of peptide are required for each determination.

Ion exchange chromatography on resin-coated chromatoplates (Dévényi et al., 1971) appeared to be suitable to monitor the process of digestion. By this method 0.5 to 1 nmole of amino acid can be unambiguously identified in a one-dimensional run, thus it may provide the basis for a convenient micro-technique.

Into hole A of the plexiglass rack shown in Fig. 1 the solution of the peptide to be analyzed was pipetted (25 to 50 nmoles, in 50 μl 0.1% ammonium bicar-

bonate, pH 8.5). A mixture of carboxypeptidase A and B (Sigma preparations, DFP-treated enzymes, specific activity, A: 60 U/mg, B: 230 U/mg) was prepared in distilled water which contained 2 U of both carboxypeptidase A and B per ml. From this mixture 5 μl was added to the peptide solution, properly mixed, and a 10 μl sample was immediately withdrawn and added to 10 μl of 0.1 *N* HCl in hole B (30 sec. sample). Hole A was then closed with parafilm and the rack was

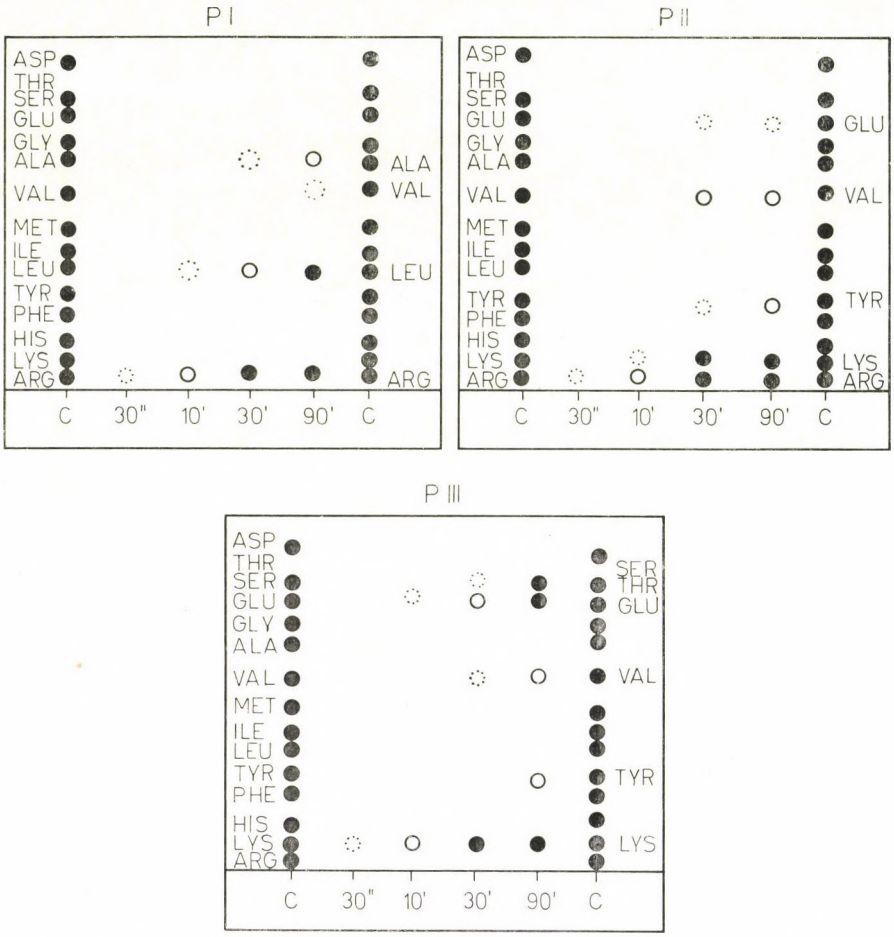

Fig. 2. Carboxypeptidase digestion of peptides isolated from the tryptic hydrolysate of rabbit muscle aldolase. About 25 nmoles of peptide has been digested in 50 μl of ammonium bicarbonate (0.1%, pH 8.5) with a mixture of carboxypeptidase A and B. Samples of 10 μl were taken at 0.5, 10, 30 and 90 minutes, the digestion was stopped by the addition of 10 μl 0.1 *N* HCl. After drying in vacuo the samples were dissolved in 10 μl of 0.01 *N* HCl and applied to a Dowex 50×8 type ion-exchange resin-coated chromatoplate. Chromatography was performed in citrate buffer, pH 3.3, at 50° for 4 hours. P I: . . .(Val).Ala.Leu.Arg; P II: . . .(Glx).Tyr.Val.Lys.Arg; P III: . . .Tyr.Val.Thr.Glx.Lys. C: Control mixture

placed in a 37° thermostate. Samples (10 μl each) were taken after 10, 30 and 90 minutes and added to 10 μl of 0.1 *N* HCl in holes C, D and E, respectively. At the end of digestion the samples were dried in vacuo, redissolved in 10 μl of 0.01 *N* HCl, and applied as a point onto a 20 × 20 cm Dowex 50x8 type (Na^+ cycle) ion-exchange resin-coated chromatoplate.* Ascending chromatography was performed at 50° and pH 3.3, in 0.4 M Na^+, 0.4 M citrate buffer (Dévényi et al., 1971), for 4 hours. At the end of the run the chromatoplate was dried and developed with collidine-ninhydrine solution.

Fig. 2 shows the results of the carboxypeptidase digestion of three peptides isolated from rabbit muscle aldolase. By carboxypeptidase digestion the following C-terminal sequences were determined:

. . .(Val).Ala.Leu.Arg	P I
. . .(Glx).Tyr.Val.Lys.Arg	P II
. . .Tyr.Val.Thr.Glx.Lys	P III

The validity of these sequences was checked by the analysis of the chymotryptic digest of the peptides.

The method described renders unambiguous, rapid and sensitive identification possible from a small amount of peptide sample.

By ion-exchange chromatography on resin-coated chromatoplates almost all amino acids can be unequivocally identified in a one-dimensional run. The resolution of threonine and serine might constitute a problem,** and difficulties may be encountered in distinguishing asparagine and glutamine from aspartic acid and glutamic acid, respectively.

Including the time required for preparations, the analysis of the C-terminal sequence of a peptide up to at least four amino acids takes about 6 hours. A great number of peptide samples can be simultaneously analyzed by this method, which is unfeasible with the amino acid analyzer.

The above technique is especially advantageous if only small peptide quantities are available owing to preparative difficulties. By this method the position of at least four amino acids in the C-terminal sequence can be conclusively identified from 25 nmoles of peptide. The same technique can be applied in degradations with leucineaminopeptidase from the N-terminus; the combined application of the two methods enables the sequence analyst to work on the micro-scale.

Thanks are due to Dr F. B. Straub for the encouragement and continuous interest. The excellent technical assistance of Mrs Clara Lendvay and Mrs Judit Báti is gratefully acknowledged.

* In some experiments IONEX-25 SA chromatoplates (Macherey & Nagel, Düren, West Germany), which have similar properties, were used.

** Peptide P III did not contain serine, therefore evaluation was unambiguous.

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Thin-Layer Ion-Exchange Chromatographic Screening Test for Aminoacidemias in Blood Samples Dried on Filter Paper

(Short Communication)

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(Received June 7, 1972)

Several methods have been elaborated for the screening and early diagnosis of congenital disturbances of amino acid metabolism. These screening methods can be divided into two major groups: A) microbiological methods such as the Guthrie test (Guthrie, Susi, 1968) and B) chromatographic methods such as paper or thin-layer chromatography (Clow et al., 1969; Stuber, 1969; Plöchl, 1968; White, 1968). The Guthrie test needs a microbiological laboratory and is almost specifically devised for the detection of hyperphenylalaninemia only. In the case of paper or thin-layer chromatography two dimensional run is necessary which is a time-consuming process and the identification is sometimes uncertain.

The recently developed thin-layer ion-exchange chromatography on resin-coated chromatoplates is a simple method for the *one-dimensional* separation and identification of amino acids (Dévényi, 1970; Hrabák, Ferenczi, 1971; Dévényi et al., 1971a, b; Ferenczi et al., 1971). The method is suitable for the examination of physiological fluids as plasma, serum, urine and cerebrospinal fluid (Dévényi, 1970; Hrabák, Ferenczi, 1971).

Thin-layer chromatography on Dowex 50×8 type resin-coated chromatoplates shows a clear analogy to the amino acid analyzer technique. On these chromatoplates the separation of amino acids can be carried out with essentially the same sodium citrate buffers which are commonly used in column chromatography. For the separation of aromatic and basic amino acids sodium citrate, pH = 5.23, Na^+ = 0.35 N, the same as used in the analyzer technique, was found to be a suitable buffer (Dévényi, 1970). For the one-dimensional separation of amino acids usually present in an acid hydrolysate, sodium citrate pH = 3.3, Na^+ = 0.4 M, citrate = 0.4 M was found to be suitable at 45 °C (Dévényi et al., 1971b). Tryptophan could be separated in sodium citrate pH = 6, Na^+ = 1.5 M buffer (Dévényi et al., 1971a). In this system tryptophan shows the lowest R_f value.

In the present paper we report this new method, as adopted to mass screening purposes for aminoacidemias in blood samples dried on filter paper.

About 50 μl capillary blood obtained by heel prick is plotted on Whatman No. 1 filter paper strip and dried afterwards. The diameter of the dried spot should be about 15 mm. This spot is punched to 5–6 small discs and put in one

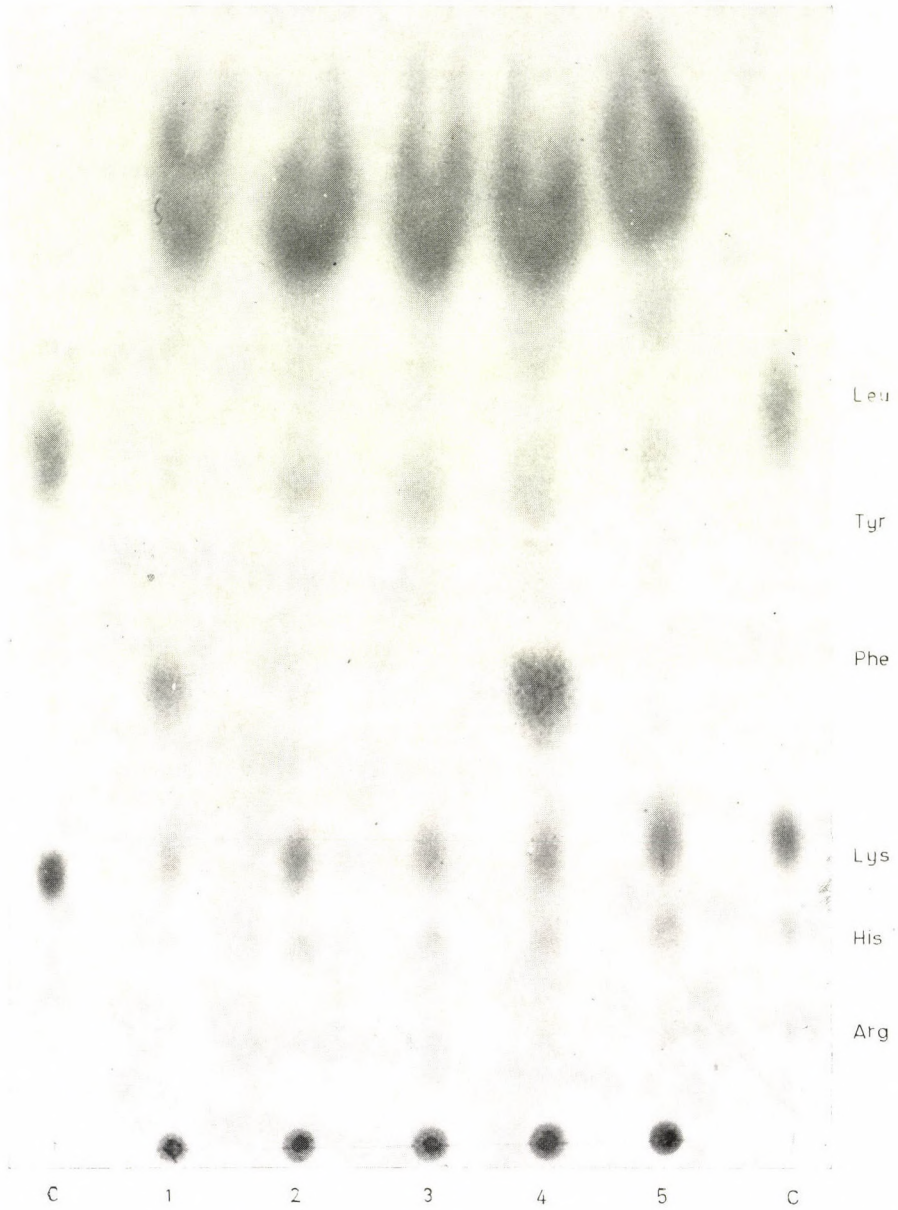

Fig. 1. Thin-layer ion-exchange chromatography of normal and phenylketonuric blood samples. Eluting buffer: sodium citrate, pH 5.23, Na^+ = 0.35 N. C = control amino acid solution containing 2 mg/100 ml Arg, His, Phe, Tyr and 10 mg/100 ml Lys and Leu. Samples 1 and 4 = phenylketonuric blood samples. Samples 2, 3 and 5 = normal blood samples

test tube. To each tube 0.1 ml of 0.1 *N* HCl in 95% ethanol is added. The tubes are sealed with Parafilm and allowed to stand overnight at room temperature. This time is sufficient for the elution of amino acids. From the content of the tubes, about 20 μl of the eluate is transferred by means of a capillary to the chromatoplate (IONEX 25 SA, Na^+-cycle, Macherey, Nagel and Co. Düren, GFR).

To detect the majority of possible specific aminoacidemias it is suitable to develop the chromatoplates in a pH 5.23, $Na^+ = 0.35$ N, aequous sodium citrate buffer solution (Dévényi et al., 1971b). The developed chromatogram is stained with ninhydrin spray (Dévényi et al., 1971b).

Fig. 1 demonstrates the chromatograms of normal and phenylketonuric sera. No. 1 and 4 show the presence of hyper-phenylalaninemia; (the quantitative analysis rendered a value of 8 and 14 mg/100 ml, respectively, by the automatic amino acid analyzer technique).

The advantages of the described method are the following:

1. The blood samples can be dried on filter paper, so that collecting, storing and mailing is very simple.

2. The method used for the elution of amino acids is suitable for mass screening programs.

3. The 0.1 *N* HCl in ethanol used for the elution eliminates the previous deproteinization of the samples.

4. The separation of amino acids by *one dimensional* thin-layer ion-exchange chromatography ensures unambiguous identification.

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Role of Histidyl Residues in the Activity of Porcine Pancreatic Amylase

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Four of eight histidines of native amylase molecule reacted with diethylpyrocarbonate (DEP) which did not alter the gross conformation of the protein. Reaction of two histidines with DEP resulted already in a practically complete abolition of amylase activity. The modified enzyme was still able to bind substrate analogs. Thus, it appeared that in amylase the catalytic (histidyl) residues and the substrate binding side chains are located relatively far from each other.

Introduction

In a previous work (Elődi et al., 1972) it was found that tryptophyl side chain(s) may be involved in the binding of substrate or substrate analogs by porcine pancreatic amylase. The binding appeared to be constant over a wide pH range, between pH 4.5 and 9.0, whereas the hydrolysis of substrate by the enzyme exhibited a pronounced pH-dependence in this range (Elődi et al., 1972a). The pH-activity relationship indicated that the hydrolysis of polysaccharide substrate depends on side chain(s) of about pK 6, possibly histidyl residues. Therefore we studied the effect of histidine modification by diethyl-pyrocarbonate* (Ovádi et al., 1967) which converts histidyl side chains to carbethoxy-histidine.

Materials and methods

Twice recrystallized porcine pancreatic amylase (Hatfaludi et al., 1966) was dissolved in 50 mM phosphate buffer, pH 5.8, containing 10 mM sodium chloride and 1 mM calcium chloride to 10 to 40 μM protein concentration and was treated at 20° with diethylpyrocarbonate (Schuchardt, GFR) dissolved freshly in 95% ethanol. The concentration of diethylpyrocarbonate varied between 0.25 to 6 mM. The reaction of diethylpyrocarbonate appeared to be specific for histidine under the experimental conditions applied. The formation of carbethoxy-histidine was followed spectrophotometrically and the number of modified histidyl residues was calculated by using a molar extinction coefficient of

$$\Delta\varepsilon_{240} = 3200$$

* This compound is also called ethoxyformic anhydride (Melchior, Fahrney, 1970).

(Ovádi et al., 1967). Calculations were based on a molecular weight of 52 000 (Závodszky, Elődi, 1970; Cozzone et al., 1970).

The perturbation difference spectra induced by the addition of substrate analogs were recorded as described earlier (Elődi et al., 1972). Enzyme activity was assayed at 37° by measuring the change in iodine-starch colour according to Smith and Roe (1949).

Maltose was a product of Reanal (Hungary), maltotriose and maltotetraose were obtained from Koch-Light Lab. Ltd. (England). β-cyclodextrin was purchased from Schuchardt (GFR). N-acetylhistidine ethyl ester was a product of Calbiochem (USA).

Results and discussion

Porcine pancreatic amylase contains 8 or 9 histidine residues (Straub et al., 1970; Cozzone et al., 1970a). In the presence of 8 M urea containing 10 mM ethylenediamine tetraacetate the histidines of the protein reacted freely and 7.8 ± 0.6 moles carbethoxy-histidine per mole enzyme were detected. Upon addition of increasing amounts of diethyl-pyrocarbonate to amylase in the absence of urea the number of carbethoxylated histidyl side chains increased but even at a relatively high concentration of the reagent (6 mM) only 3.8 ± 0.2 moles of histidine per mole enzyme were modified without previous alteration of the protein structure. This observation may indicate that about half of the histidyl side chains are inaccessible to the reagent. Two out of four histidines reacted faster with diethylpyrocarbonate than the others. The second order rate constants of carbethoxylation, k, were 21 and 1.2 M^{-1} min^{-1} for the fast and slowly reacting residues, respectively. For comparison, a rate constant of $k = 125$ M^{-1} min^{-1} was determined for N-acetylhistidine ethyl ester under similar conditions.

The effect of carbethoxylation on enzyme activity was tested in the following way. To the protein solution diethylpyrocarbonate was added and the increase in the absorption difference at 240 nm was recorded in time against a control protein solution. At intervals samples were withdrawn and were immediately diluted 500-fold with Tris-HCl buffer, pH 7.4, containing 1 mM calcium chloride and the activity was assayed. The dilution prevented the modification of further histidines.

The modification of histidyl side chains resulted in a decrease of enzyme activity. Fig. 1 shows that there is a linear relationship between the number of histidines reacted and the decrease in enzyme activity. Carbethoxylation of two histidines almost completely abolished the enzyme activity. A second order rate constant of $k = 15$ M^{-1} min^{-1} was found for the loss of activity due to carbethoxylation of histidines, a value near that found for the reactivity towards diethylpyrocarbonate of "fast" histidines, 21 M^{-1} min^{-1}. This suggests that there is a direct correlation between the modification of two fast histidines and the decrease of catalytic function of the enzyme.

The totally inactive enzyme containing 2.5 carbethoxy-histidines could almost fully reactivated by incubation with neutral 0.2 M hydroxylamine at 20 °C for one hour (see Table). The inhibition could also be reverted by adjusting the protein solution with ammonium hydroxide to pH 10 followed by incubation at 20 °C for 2 to 3 hours. Incubation at alkaline pH, however, also diminished

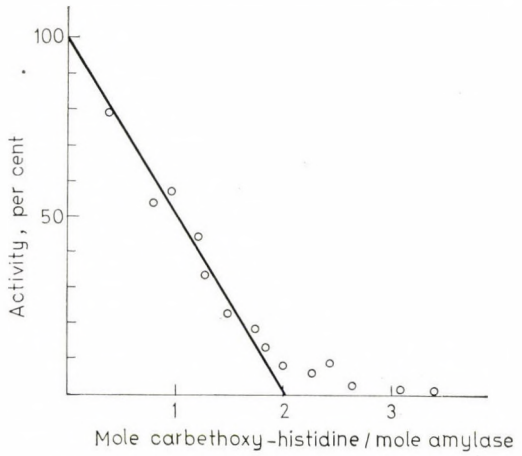

Fig. 1 Effect of modification of histidyl residues in amylase. 20 μM amylase dissolved in 50 mM phosphate buffer, pH 5.8, containing 10 mM sodium chloride and 1 mM calcium chloride was treated with 6 mM diethylpyrocarbonate at 20° C and the modification of histidines was followed spectrophotometrically at 240 nm. At intervals samples were withdrawn and diluted 500-fold with 10 mM Tris-HCl buffer, pH 7.4 and were tested for enzyme activity

the activity of the untreated control sample with about 35%. Both hydroxylamine and alkali treatments removed almost completely the carbethoxy residues from the protein as controlled spectrophotometrically by measuring the decrease of absorption difference against a control at 240 nm.

Optical rotatory dispersion and difference spectrophotometric measurements have shown that the gross conformation of amylase, containing four modified histidines, remains unaltered. However, slight structural alterations might have occurred as a result of carbethoxylation of 2 to 4 histidyl residues as one of the two buried sulfhydryl groups of amylase became reactive towards 5,5'-dithio-bis-(2-nitrobenzoate). In the unmodified enzyme the sulfhydryl groups react with the thiol-reagent only in the presence of ethylenediamine tetraacetate.

Since the change in reactivity of the sulfhydryl group indicated slight local structural alterations in the enzyme, the question arose whether the conformation of active center was also affected by this change. This can be tested spectrophotometrically by binding studies (Elődi et al., 1972) with linear (maltose, maltotriose, maltotetraose) or cyclic (β-cyclodextrin) substrate analogs. The interaction of these compounds with the enzyme will be discussed in details elsewhere (Elődi,

Table 1

Reversibility of inactivation of amylase by carbethoxylation

Enzyme	Experimental conditions	Mole carbethoxy-histidine per mole enzyme	Activity, per cent
Control	in Tris buffer, pH 7.4	0	100
Control	in 0.2 M hydroxylamine, pH 7.4	0	95
Carbethoxylated enzyme	in Tris buffer, pH 7.4	2.5	0
Carbethoxylated enzyme	in 0.2 M hydroxylamine, pH 7.4	0.2	91
Control	in ammonium hydroxide, pH 10	0	65
Carbethoxylated enzyme	in ammonium hydroxide, pH 10	0.2	60

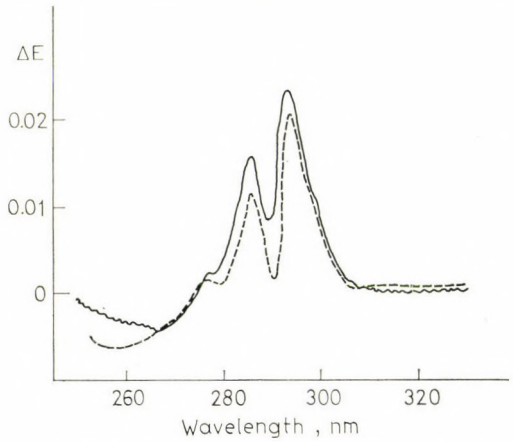

Fig. 2. Difference spectra of amylase induced by β-cyclodextrin. 12 μM amylase solution in 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM calcium chloride and 1.7 mM β-cyclodextrin recorded against a reference without cyclodextrin. Solid line: control; dotted line: amylase containing 2.5 carbethoxylated histidines per mole enzyme

Móra, in preparation). The perturbation difference spectra induced by 1.7 mM β-cyclodextrin with the untreated and carbethoxylated enzyme are shown in Fig.2. From the comparison of the two spectra we may conclude that the inactive enzyme can still bind the substrate analog. The dissociation constants of enzyme substrate analog complexes, K_S, were 250 and 400 μM for the untreated enzyme and for the enzyme containing 2.5 carbethoxy-histidines, respectively, as calculated from the absorption difference at the maximum of difference spectra induced by the substrate analog. This indicates that the enzyme substrate analog interaction was only slightly altered by modifying histidines.

Similar results were obtained with the linear substrate analogs. Thus, for example, dissociation constants of 13 and 60 mM were obtained for the maltose complexes of the native and modified enzymes, respectively. It also has to be mentioned that 50 mM maltose did not influence the reactivity of histidines towards diethylpyrocarbonate. We concluded from these observations that the modification of histidines did not significantly alter the conformation of the active center.

According to Robyt and French (1970) the active center of amylase occupies a relatively large area. It is then conceivable that the tryptophyl side chain(s) participating in the binding of substrate analogs (Elődi et al., 1972) may be located relatively far from the functional histidines, at least at a distance as great as one glucose unit. Another reasonable possibility is that the carbethoxy residue on the histidyl side chains is oriented so that it does not interfere with the binding of substrate analogs.

Since the modification of two histidines resulted in the complete abolition of activity, it remains to be seen whether both side chains are located in the active center. Binding studies with maltose and limit dextrins (Loyter, Schramm, 1966) suggested that pancreatic amylase has two binding sites and, probably, two active centers. According to the considerations of Ray and Koshland (1961), however, very accurate kinetic determinations of both activity loss and modification of side chains are necessary to decide between the two alternatives, i.e. to ascertain whether one or two histidines participate in the catalytic function of amylase. The accuracy of the analytical procedures applied in these investigations does not permit to settle this question, thus it still remains a subject of further studies.

The discussion of the manuscript with Dr G. Szabolcsi was most helpful. The excellent technical assistance of Mrs P. Tóth is greatly acknowledged.

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The Effect of cAMP on Phospholipid Metabolism

(Short Communication)

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The direct influence of cAMP* on some membrane structure and function may be supposed to be the first step during the course of its known metabolic effects.

In the present work the effect of cAMP on the lecithin-lysolecithin system of rat lung tissue was investigated in vitro. No data can be found in the literature concerning the relation between cAMP and phospholipase A enzyme activity. Our previous experiments have suggested the possibility of such a relation (Imre, 1970).

In our experiments enzyme activity was measured not by classical methods using exogenous substrate, but by measuring the activity of lung phospholipase A acting on its own substrate, i.e. the phospholipids of lung (alveolar) membranes. The tissue homogenate was incubated for 60 min and the changes of the quantity of the substrate and reaction product that occurred upon addition of cAMP and theophyllin were compared with those obtained in controls without additives.

Rat lung tissue was homogenized in a Potter–Elvehjem homogenizer, ice-cold phosphate-saline medium containing human serum albumin (5 g/100 ml) and (as enzyme activator) 10^{-2} M Na-deoxycholate (Mosinger, Vaughan, 1967) (1 g tissue + 3 ml phosphate-saline medium).

The homogenized tissue was centrifuged at $1600 \times g$ for 10 min and the supernatant was incubated in a thermoshaker at pH 7.4, 38° for 60 min.

After incubation lipids were extracted with four volumes of $CHCl_3 : CH_3OH = 2 : 1$ mixture and from the lower chloroform phase the lecithin and lysolecithin contents were determined by the thin layer chromatographic method of Rossi (Rossi et al., 1965). Lecithin and lysolecithin were expressed in μg P and related to 1 g of protein. Protein was determined by the method of Lowry (Lowry et al., 1951), phosphorus by the method of Fiske and Subbarow (Fiske, SubbaRow, 1925).

Theophyllin slightly decreased the quantity of both lecithin and lysolecithin, whereas cAMP alone significantly lowered the lecithin content. If cAMP and theophyllin were both added the lecithin content was decreased and the lysoleci-

*Abbreviations: HSA = Human serum albumin, cAMP = Adenosine 3′-5′-cyclic phosphate.

Table 1

Changes of lecithin and lysolecithin content in rat lung tissue

Reaction mixture in 2 ml: Supernatant of lung tissue homogenate (50%) in phosphate-saline medium [0.154 M NaCl : 0.1 M Na_2HPO_4 (pH 7.4) = 10 : 1] containing HSA (5 g/100 ml) and 10^{-2} M Na-deoxycholate.

	Lecithin μgP/hr/g protein	Lysolecithin μgP/hr/g protein	Lecithin / Lysolecithin	Sum of lecithin and lysolecithin μgP
Control	6060 ± 120	1452 ± 71	4 : 1	7512
cAMP (10^{-3} M)	3984 ± 483	1530 ± 98	2.6 : 1	5514
Theophyllin (10^{-4} M)	4890 ± 508	1308 ± 121	3.7 : 1	6198
cAMP + Theophyllin	3984 ± 402	1884 ± 130	2.1 : 1	5868

± = The values are the averages of 11 groups, each consisting of 3 animals.

thin level was significantly raised ($p < 0.01$). This is an unequivocal sign of the increase of phospholipase A activity.

The altered ratio of lecithin-lysolecithin also reflects this increased activity. The decrease of the total amount of these phospholipids indicates that some change of lecithin synthesis or increase of lysolecithin decomposition play a role in the development of this phenomenon.

The very pronounced effects found when both theophyllin and cAMP were added may be explained by the fact that theophyllin prolongs and increases the effect of cAMP through the inhibition of the phosphodiesterase enzyme which splits cAMP (Butcher, Sutherland, 1962).

It is true, we cannot say that the above alterations are due to changes in the activity of a single enzyme. In the complex system investigated by us only the final result of the correlated changes in lecithin and lysolecithin can be detected. However, it seems probable that in the development of these changes phospholipase A plays a major dominant role.

This hypothesis is supported by our experiments according to which Na-deoxycholate (10^{-2} M) as an activator of phospholipase A appears to be necessary to obtain these changes (Imre, unpublished results).

In conclusion, during the course of a 60 minute incubation of homogenized rat lung tissue in vitro cAMP together with theophyllin produced a significant lowering of the lecithin level and a parallel rise of the lysolecithin level. These effects are related to the activation of phospholipase A.

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Relationship between Structure and Function in the Radiation Injuries of T7 Phages*

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The authors analyzed the radiation injury process from the absorption of the UV photon until the biological manifestation of radiation injury. For the analysis the results of their earlier kinetic model on the radiation injury of T7 phages and the data in the literature on the molecular properties of T7 phages are used. By the analysis they conclude that the injurable places assumed in the model are the cytosine clusters of T7-DNA, which are functionally the initial and the terminal sites of the transcription, respectively. The one hit kinetics is based on the information content of T7 phages concerning non-lysogene functions.

Introduction

In the elucidation of the morphological and radiochemical properties of in vivo radiation injuries the studies of radiation injury of bacteriophages have already led to numerous results. These experimental objects, usually considered as the model of cellular radiation injuries and showing even the simplest life processes only under appropriate conditions, proved very convenient to check on our in vitro radiobiological experiences since they are, as biological material, macromolecules. Recently, every or almost every function of a few simple bacteriophages (RNA phages, T3, T7) and, more or less, also the regulatory mechanism of these functions have been explored (Kelly, Thomas 1969; Summers, Szybalski, 1968; Studier, 1969; Summers, 1970; Karczag, Rontó 1970). This fact enables us to return to some problems of the radiation injuries of T7 phages studied by us earlier in detail, to compare them with the relevant molecular biological data and, knowing this, to make indirect conclusions as to the different phases of radiation injury and the relationship between the injury of the biological structure and of the function.

The present paper deals with some consequences of our radiation kinetic model constructed on the basis of the UV inactivation dose-effect curves of T7 phages (Rontó et al., 1967; Tarján, Rontó 1970). We wanted to give them a further biological interpretation just in order to reveal the connection between the injuries of the structure and of the function.

* Based on a paper read at the VIth Congress of the Hungarian Biophysical Society, Pécs.

1. The nature and localization of the induced photoproduct

For irradiation the UV light of a germicidal lamp was used. We have shown the $\lambda = 254$ nm mercury line to be the effective one in the spectrum of the lamp (Rontó, 1969). Recently it has been proved that this wavelength does not cause practically any breaking of a normal phage-DNA strand (Hotz, 1971); thus, at irradiation, one has to reckon only with the creation of the UV photoproducts of the bases. It is known from in vitro studies that, out of the nucleotide bases, the UV light mainly affects the pyrimidine bases, by creation of dimers (McLaren, Shugar, 1964; Dellweg, 1968).

A necessary condition for dimer production is that at least two pyrimidine bases be side by side within a polynucleotide chain. And it is characteristic of the dimer that the same UV photon which has caused dimerization, may cause monomerization as well if absorbed by a dimer (Setlow, 1966; Dellweg, 1968). The probability of dimerization and monomerization caused by the absorbed photon is, of course, different.

In our earlier works we showed qualitatively that the UV inactivation dose-effect curve of T7 phages was modified by direct photoreversion. Quantitative evaluation of our experimental data revealed that the absorbed 254 nm photon caused reactivation of a phage after single injury with a probability by one order of magnitude lower than the probability of injuring an intact phage (Rontó et al., 1967; cited by Dertinger, Jung, 1969).

Accepting that the chemical basis of the direct photoreversion is a dimer–monomer conversion, one can conclude that the injury in the T7-DNA means photochemically the production of *pyrimidine-dimers.*

To reveal the *more exact localization and nature* of the injury one must consider the primary structure of the T7-DNA. Summers and Szybalski (1968) studied the composition of DNA in numerous microorganisms. They stated that in all DNA one part of the pyrimidine bases could be generally found in the form of clusters (containing 15 to 40 bases). But while the mentioned clusters could be found evenly distributed in both strands of most of the studied DNA, in the T7-DNA they were found only in one of its strands. Another characteristic feature of the T7-DNA pyrimidine clusters is that they are consisting of cytosine alone; the thymine bases are distributed randomly along the two strands.

Since in the cytosine clusters there are given the conditions for dimer production one must conclude that with great probability, the cytosine clusters are injured in the T7 phage: cytosine dimers are produced in them.

Summers determined also the number of cytosine clusters present in one T7-DNA molecule and obtained a value of 30 to 70 (Summers, Szybalski, 1968; Szybalski et al. 1966). Based on our radiation kinetic model we estimated the number of injurable places (s) to be about 100 or higher (Rontó et al., 1967). These two data do not contradict each other and from the above facts one may conclude that the *cytosine dimers* produced in T7 phages are *localized in the cytosine clusters*; the number of clusters is in good agreement with that of the injurable places in the radiation kinetic model.

These structural elements play, thus, a particular part in the radiation injury. This conclusion of us concerning the increased injurability of the pyrimidine clusters, is in agreement with the experiences obtained by other authors on other DNA. E.g. Gumpelmayer and Altmann (1971) reported the localized strand-breaking of the pyrimidine clusters after γ-irradiations of Salmon sperm DNA.

2. Functional consequences of the structural injury

In the following the role of pyrimidine clusters in the DNA molecules will be studied; in the present case we are dealing with the T7 DNA and the possible consequences of a dimer formation in them. Since the injury does not affect the DNA strand not containing cytosine clusters, i.e. not participating in the replication, one must consider the functioning and the injury of this functioning of the minus strand to be transcripted and, within this the cytosine clusters.

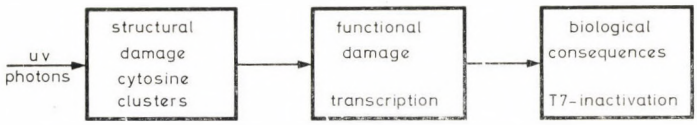

Fig. 1. The phases of the UV inactivation of T7 phages (schematic drawing)

According to the results of Szybalski et al. (1966), Szybalski (1969) and Summers and Siegel (1970), the pyrimidine clusters are the initial and terminal sites of the transcription. During the transcription no mRNA part can be correlated to these DNA parts, they only ensure by their geometrical structure the possibility for the RNA polymerase of establishing the template-enzyme interaction. It can be seen that even the slight change of the geometrical structure of initiation sites accompanying the dimer formation causes a disturbation of the start and of the end of the transcription, a feature resulting in the outfall of a gene function.

In the case of the T7 phage the outfall of one gene function leads to the inactivation of the plaque-forming ability of the phage, because the products of every gene of the T7 phage are equivalent, that is, the virulency of the phage (its plaque-forming ability) is bound to the function of all genes (Studier, 1969; Studier, Maizel, 1969). It follows from this that the biological consequence of the injury of the transcription of any gene is the inactivation of the plaque-forming ability. This condition also explains our experience on the one hit characteristics of the T7 dose effect curve.

Our results on the radiation injuries of T7 phages and our conclusions derived from them indirectly are summarized schematically in Fig. 1. The injury caused by the UV photon is localized to a concrete constituent of the DNA molecule, the cytosine clusters, the injury of the function appears at the level of tran-

scription, which finally manifests itself biologically in the absence of plaque forming ability.

We analyzed the above-sketched process of radiation injury in an indirect way. We are of the opinion that beside the direct investigations, the further exploration of the chain of processes may give very important data concerning the radiation injury of RNA phages and the nature of radiation injuries caused by far UV light. Our studies in this direction are now in progress.

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Time-relations of Initial Volume Decrease and Contraction in Frog Muscles

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During indirect stimulation of unstretched frog gastrocnemius muscle the initial volume decrease and twitch were examined simultaneously in temperature ranges from 4 to 24 °C. It was found that, on the basis of the temperature-dependence of the initial volume decrease and the time data of the twitch of the unstretched muscle, no parallelism could be shown between the two phenomena.

Introduction

Several authors examined the volume change of the unstretched or stretched muscle, parallel with other phenomena of the activity of the muscle, in order to elucidate the connection between volume change and the electric and mechanic phenomena of muscle activity.

The simultaneous registration of initial volume decrease and action potential as well as of volume decrease and contraction, resp., in unstretched muscles was first made by Ernst (1963) in 1928. In these works he stated that the changes in the amplitude of the initial volume decrease and action potential are roughly parallel in one series of fatigue and, that the volume decrease precedes contraction. By the simultaneous registration of the temperature dependence of the amplitude of the intial volume decrease and the action potential, Ernst et al., (1954) came to the conclusion that the time course of the two effects is parallel to each other. Aradi and Schäffer (1971) obtained similar results concerning the temperature dependence of the relative time data of the two effects.

By investigating the volume decrease and the action potential separately in the isotonic activity of frog gastrocnemius muscle, Meyerhof and Möhle (1935) found the volume decrease at low temperature to become slower than the action potential and that this slowing corresponds to the change of the speed of contraction. In the opinion of Baskin and Paolini (1965) the time data of volume decrease and tension are the exponential functions of the temperature. The experiments of Baskin and Paolini (1966) on frog sartorius also led to the conclusion that the volume decrease and the development of tension have a similar temperature dependence. It was the aim of the present experiments to determine, by parallel investigations of the phenomena, the time relation of the initial volume decrease and contraction of the unstretched muscle as well as its action potential and contraction in the function of time.

Methods

The experiments were performed on unstretched frog (Rana esculenta) ischiadicus-gastrocnemius preparations.

The exprimental apparatus used essentially corresponded to the apparatus described previously in detail (Aradi, 1970; Aradi, Schäffer, 1971); a semi-schematic diagram of the apparatus used for registrating the contraction is shown in Fig. 1.

The mechano-electric transducer registering the contraction of unstretched muscle essentially consisted of two parallel platinum armaments, submerged into

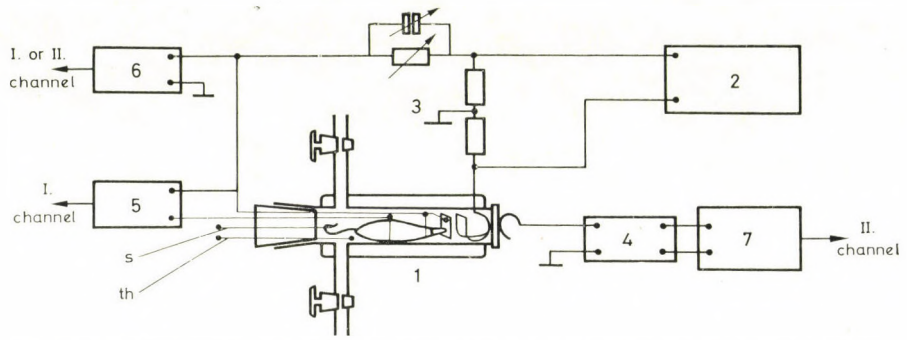

Fig. 1. Scheme of the apparatus measuring the temperature dependence of the initial volume decrease, the action potential and the contraction of the muscle. (1) Muscle- chamber with coolant jacket (s = stimulating electrodes, th = thermoelement), (2) 10 kHz oscillator, (3) measuring bridge, (4, 5) low pass filters, (6) high pass filter, (7) pre-amplifier

the Ringer's solution of the muscle chamber; one of them was directly fixed to the tendon, and one of the electrodes used for recording the action potential was used as an outlet; a lengthening of the earth electrode of piezo-electric quartz crystal served as the other armament. The change of impedance appearing during the twitch of the muscle between the two armaments was registered as a quantity roughly proportional to the contraction. The above-mentioned armaments were connected into a measuring bridge (3) fed with the sign of a 10 kHz oscillator (2) which was equilibrated before measuring.

The 10 kHz measuring voltage appearing in the sign of volume decrease and action potential was suppressed by low pass filters (4 and 5), and the possible disorder appearing in the sign of contraction was suppressed by a high pass filter of 10 kHz limiting frequency.

One channel of the double channel oscilloscope transmitted the sign of the action potential; the sign of volume decrease was transmitted to the second channel after interposing a pre-amplifier (7); the sign of contraction was passed on by Channel I or II depending on whether we wanted to record it simultaneously with the action potential or simultaneously with the volume decrease.

The preparation was indirectly stimulated with rectangular impulses of IV amplitude and 0.2 ms duration.

The experiments were performed at 4, 8, 12, 16, 20 and 24 °C; the temperature was regulated and measured as described previously (Aradi, Schäffer, 1971).

Results

Fig. 2 shows a series of experiments which was performed at temperatures of 4, 8, 12, 16 and 20 °C. In the oscillograms of column "a" the sign of contraction can be seen above and that of volume decrease below; on the oscillograms of

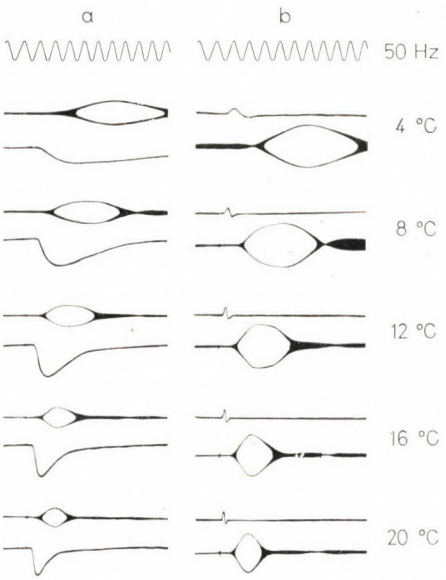

Fig. 2 a—b. a) Contraction of frog gastrocnemius (above), and initial volume decrease (below); b) its action potential (above) and contraction (below), at temperatures of 4, 8, 12, 16 and 20 °C

column "b" the sign of action potential can be seen above and that of contraction below.

For evaluation the time data of the three phenomena were measured at the moment of stimulus break in: these phenomena were the time of the maximum volume decrease (t_v), the whole time course of the biphasic action potential (t_a) and the latency time of the contraction (t_k). The above-mentioned time data are shown in oscillograms recorded at a temperature of 20 °C (Fig. 3).

Supposing that the measured time data change proportionally with the exp $\left(\frac{B}{T}\right)$ function — where T is the absolute temperature —, a straight line was fitted

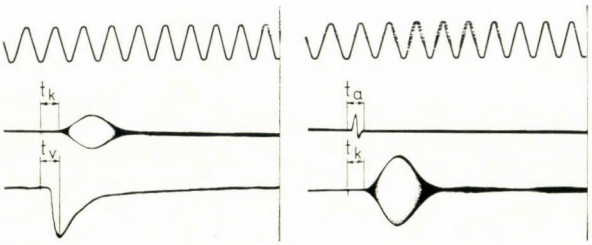

Fig. 3. Time data measured for the purpose of evaluation as related to the moment of stimulus break in. t_v = duration of maximum volume decrease, t_a = duration of the whole course of action potential, t_k = time of latency of contraction

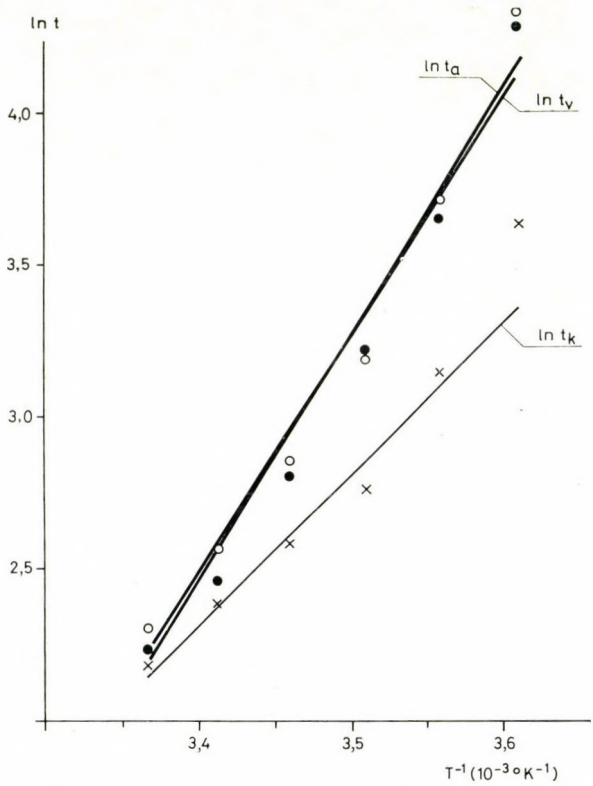

Fig. 4. Logarithm of the measured data of initial volume decrease (○), action potential (●) and contraction (×) plotted against the reciprocal of the absolute temperature

to the logarithm of the time data with the method of weighted smallest squares, using the standard deviation of the time data as weighting factor. Fig. 4 shows the logarithm of the time data plotted against the absolute temperature as well as the straight lines fitted to it.

The analytic forms of the straight lines fitted to the logarithm of the time maximum volume decrease (t_v), the whole time course of action potential (t_a) and the time of latency of contraction (t_k) are:

$$\ln t_v = (7.81 \pm 0.50)10^3 T^{-1} - (24.07 \pm 1.03) \quad (1)$$

$$\ln t_a = (8.06 \pm 0.58)\ 10^3 T^{-1} - (24.94 \pm 1.12) \quad (2)$$

$$\ln t_k = (4.97 \pm 0.55)\ 10^3 T^{-1} - (14.59 \pm 1.06) \quad (3)$$

Our experimental results read from the presented series of experiments and from Equations (1), (2) and (3) can be summarized as follows:

1. In the temperature range examined the latency time of the contraction is significantly longer than that of the volume decrease and of the action potential at every points of temperature; so, the latter two effects precede the twitch of unstretched muscle.

With the aid of the fitted straight lines – taking also the standard deviation of parameters into consideration – no parallelism can be demonstrated between the temperature dependence of volume decrease and twitch while our parameters obtained for the temperature dependence of volume decrease and the action potential corroborate our previous results (Aradi, Schäffer, 1971).

Among many thousands of picture-records made in the earlier (Aradi, Schäffer, 1971) and in our present experiments there is not a single one showing a volume increase.

Discussion

Our experimental results concerning the duration of the maximum initial volume decrease and the latency of twitch roughly correspond to the data in the literature inasmuch as both values decrease together with decreasing temperature. But significant differences appear in the quantitative data of the two effects.

According to Meyerhof and Möhle (1933), the volume change and the shortening begin at the same time; but their time data are in accordance with all the results of Ernst et al. (Ernst, 1963): the initial volume decrease precedes the twitch of unstretched muscle within a temperature range of 4 to 24 °C.

While Meyerhof (1935) experienced that during the "isotonic" activity of frog gastrocnemius muscle the volume decrease becomes slower corresponding to the change of the contraction speed at a low temperature, we could not demonstrate such a parallel change in the time dependence of volume decrease and twitch in most of our experiments.

Our above data obtained for the temperature dependence of the time data of volume decrease and action potential corroborate the results of Ernst et al. (1954), as well as those of Aradi and Schäffer (1971), according to which the time course of the two effects has a parallel temperature dependence.

Beside corroborating the parallel change of the time data of the initial volume decrease and the action potential in the function of the temperature, our

experimental data indicate that, when only the temperature dependence of the time data is concerned, no connection can be found between the initial volume decrease and twitch of the striated muscle.

The authors wish to express their thanks to Professor E. Ernst for his thematical and methodological initiative.

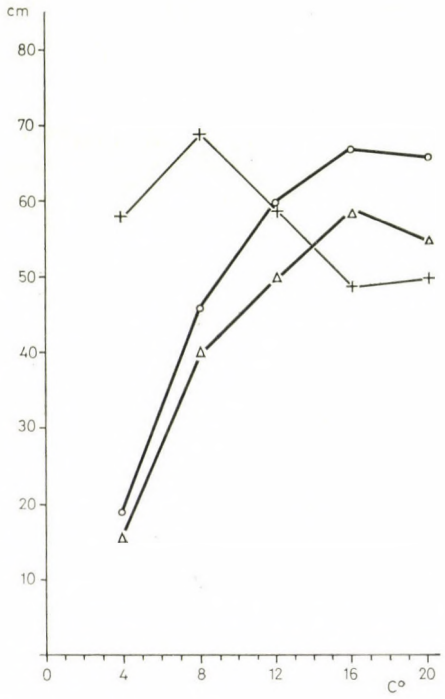

Fig. 5. Amplitudes of the action current (o), volume decrease (△) and contraction (+) expressed in cm-s plotted against the temperature

Addendum

Referring to Baskin's and Meyerhof's papers my co-workers also applied the s.c. van't Hoff coefficient Q_{10} in their previous (Aradi, Schäffer, 1971) and present papers comparing the time data of action current, initial volume decrease and muscle contraction. However, the very great differences in the methods of recording these effects make any such quantitative evaluations doubtful. Accordingly, the last item of this paper was – on my advice – left out. Instead Fig. 5 gives a *qualitative impression* of how the amplitudes of these three effects change between 4 and 20 °C. The numerical data used for the curves in Fig. 5 are the mean values calculated from the measured values of the curves recorded in two series

of experiments. To speak more exactly requires to perform more precise experimental work; notwithstanding the number of papers producing splendid mathematical formulations irrespective of any biological reality seems to be increasing today.

E. Ernst

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Non-electromagnetic Holography and Its Impact on Bio-medical Research and Clinical Practice

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The scope of our presentation was to show that the holographic principle, which provides the means for recording with square law detectors, the phase information of an object wave can be applied not only to information processing procedures where electromagnetic waves are involved, but also in the case of mechanical waves. Furthermore, the concept of holography has its impact on the interpretation of biological information processing mechanism. Both ultrasonic holography and biohologrophy may have a great influence on further evaluation of medical research and clinical application.

Selected passages from the introductory talk of the Technical Group Meeting "Information Processing, Holography and Coherence" at the 1970 Annual Meeting of the Optical Society of America, Hollywood, Florida, September 28–October 2, 1970.

Introduction

The title "Non-electromagnetic holography and its impact on bio-medical research and clinical practice" was chosen for this meeting with the expectation of providing a reasonable framework within which the organization of our discussion could develop.

We can specify three principal phases in the analysis of bio-medical and especially clinical work, defining them as

a. *detection*, i.e. showing that there is "something"
b. *description* of the "something" by numeric and non-numeric parameters
c. *diagnosis*, i.e. determination of the "something".

These three principal phases are clearly analogous to the major phases involved in information processing with coherent light. However, because few clinically trained people are familiar with optical data processing techniques and vice versa, a higher level of effort has been devoted to research and development of coherent information processing in various technical fields than that expended on developing techniques for bio-medical and clinical information processing.

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The most important and fundamental contribution of holography to optical data processing is that it provides the means for recording, with square law detectors (Gabor, 1948, 1949), the phase information of an object wave. This contribution, however, was more theoretical than practical before the development of the laser, since the need for coherent optical sources was the main obstacle in the spreading of holography. The most surprising fact, however, is that coherent energy sources other than light, have been with us for a long time in the form of ultrasonic transducers, nevertheless, up till 1964/65, nobody tried to make a holo-

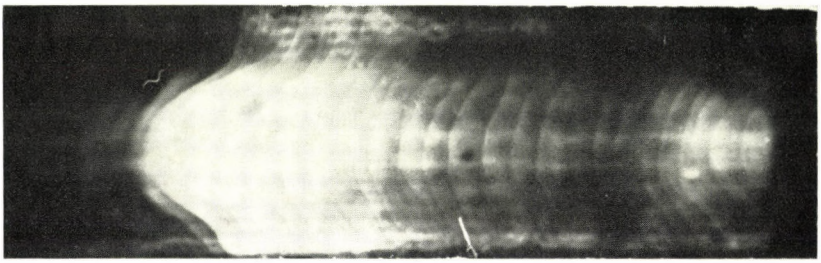

Fig. 1. Ultrasonic picture of a heat wave traveling in a plexi bar taken by time-lapse technique

gram on this basis (Greguss, 1965, 1966a). Recently we have shown (Greguss, 1969) that ultrasonic holograms have been made before the optical ones. N. Marinesco and co-workers (1933) when studying the effect of ultrasonic irradiation on photographic materials produced, involuntarily, ultrasonic holograms already as early as 1933, i.e. 15 years before D. Gabor discovered holography. And even then, scientists working in ultrasonic visualization did not recognize that sometimes their ultrasonic pictures were not useless, out-of-focus pictures but real ultrasonic holograms (Keck, 1959). The first paper on ultrasonic holograms was published in 1965 (Greguss, 1965). Fig. 1 shows one of the earliest ultrasonic holograms made at the Ultrasonic Laboratory, RSRI, Budapest.

The difficulties in making ultrasonic holograms are rather different from those encountered in making optical ones. One of these is to create an efficient and economical acoustical-to-optical energy converter. Another is to compensate for the disparity between the wavelengths of the read-in (recording) ultrasound and read-out (reconstructing) light (500 : 1). Just, therefore, those who are familiar with ultrasonics raise the question: why to extend holography as it is known in optics to sound hence both amplitude and phase information of an ultrasonic wave can be measured without the need of the technique of holography. I hope at the end of this presentation the answer to this question will be obvious.

Another question, however, should be answered now. Namely, why to try to use "non-electromagnetic" waves for information processing in bio-medical research and, especially, in clinical diagnosis? The fundamental factor is, however, not – as it could be expected – that ultrasound "sees through" opaque media

too, since X rays may do the same job, perhaps better. Ultrasounds as mechanical vibrations differ, however, from other forms of radiation in interactions with living systems; at the intensity levels used in clinical diagnosis, it is not an ionizing radiation, such as X rays. So, information is available from its use without the risk of undesirable damage. The results obtained with it are not only complementary to other diagnostic methods, but in several cases, such as in investigation of children and pregnant women, this is the only possibility to get information of diagnostic value.

Recording an ultrasonic hologram

Recording an ultrasonic hologram means to visualize the intensity distribution of an ultrasonic field which was created by the interaction of the information bearing wave front and the reference wave front. Therefore, first of all, we have to review briefly those soundfield visualizing techniques which seem to be adequate for recording ultrasonic holograms.

a) A *liquid surface* will deform due to sound pressure, and this deformation can be viewed by reflecting light from the surface. Sokoloff (1935) was the first who proposed and used this method in 1929 to detect flow in metals by transilluminating them with an ultrasonic beam in the region of several megahertzs. Since this time, several endeavours have been made to improve this idea and to get a device which could be used in every-day practice. Extensive bibliography will be found in References (Mueller, 1968; Korpel, Desmares, 1969).

b) Similarly, systems which reproduce an ultrasonic field pattern as a television-like picture are mainly based on a concept of Sokoloff (1937) who, for the first time, suggested that a piezoelectric plate on a vidicon tube could convert the sound-picture into a piezoelectric charge image. Using a moving electron beam to scan the charge image, a video signal is produced as in a vidicon. After aplification, it is applied to the modulator of the display cathode ray tube. The latter then shows the image of the object. The main drawback of this technique is that if the angle of incidence is greater than about 5°, then, most of the ultrasonic energy will be converted to shear waves and the recording will not take place, and also the angular field of view is limited for less than 18°. It is purely coincidental that the total reflection angle for sound waves impinging from water to quartz is 15° which is very close to the cut-off angle determined by the detector aperture. Due to this fact, the field of view limitation of piezoelectric detectors is sometimes wrongly attributed to total reflection.

As the light recording capacity of photographic plates may be based on different photochemical reactions, so sonographic plates could be founded on sonochemical reactions. The different approaches to develop a reliable and simple sonosensitive plate have been reviewed elsewhere (Greguss, 1966 b).

If the intensity distribution of a soundfield is recorded not in a gray scale but it is converted into a colour scale, the perceptibility of different acoustic impedances can be improved by several orders of magnitude. This statement manifests itself

very dramatically in the following sonopictures. In Fig. 1, a black and white sonopicture of a heatwave traveling in a plexi bar is shown, taken by a time-lapse technique. The ultrasonic absorption in plexi (Lucite) varies namely with temperature considerably. Fig. 2 is the colour version of the same picture transformed into colour sonogram by using the Model 702 Data Color System of the Spatial Data Systems, Inc., Goleta, California.

c) Another approach to get a visual display, and what is more, in colour, is to use the well-known effect that when an acoustic image is projected onto a suit-

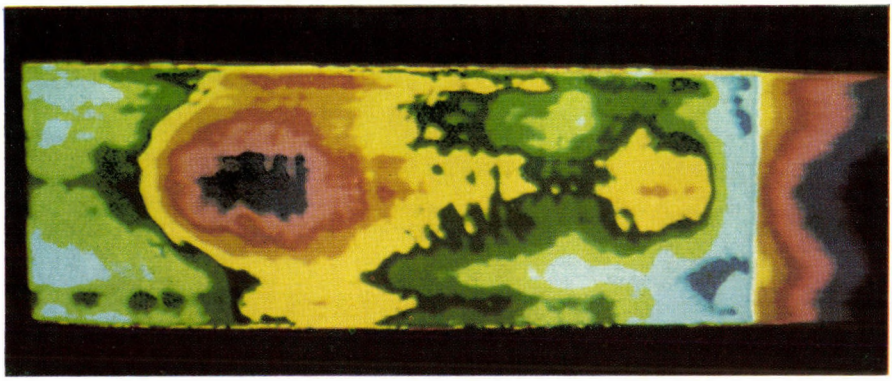

Fig. 2. Same as Fig. 1, only in pseudo-colour

able absorbing material, this image will be converted into a corresponding spatial temperature variation over the absorber. If then such a sound absorber is coated with a layer of an appropriate crystal, the spatial temperature variation in the absorber will result in a colour image with the colour indicating the sound intensities in the acoustic image. The reality of this type of acoustical-to-optical converter was first demonstrated just 20 years ago using $CoCl_2 \cdot 6H_2O$ crystals (Greguss, 1951). Recently, a good many researchers suggested to use cholesteric liquid crystals (Metherell et al., 1969). Although this idea is simple, up until now, its technical realization is not. With the assistance and support of the National Cash Register Company, we are trying to develop an ultrasonic field visualization technique based on microencapsulated liquid crystals which could be used e.g. for soft tissue differentiation. It is a well established fact that absorption properties of abnormal tissues differ from the normal ones. So e.g., a breast tumour could be detected in its early stage by measuring the ultrasonic absorption properties of the soft tissue.

The breast to be investigated would be covered with a washable black plastic which dries in a few seconds. After this, a slurry of encapsulated liquid crystals is sprayed over the black plastic. Since these liquid crystals are *not* sensitive to the surface temperature of the body, they will not show any colour changes during the procedure. Transilluminating, however, the breast with an ultrasonic

beam, the occasional presence of a tumour may be demonstrated by colour changes since there is a difference not only in the acoustic impedances but also in the ultrasonic absorption coefficients of the normal tissue and that of the tumour.

Reconstruction of ultrasonic holograms

With the brief and far not complete review of techniques suitable for the visible recording of the intensity distribution of an ultrasonic field, we wanted only to show that the main problem in ultrasonic holography is not the first step, the recording phase, but the second one, the wavefront reconstruction, due to the 500 : 1 disparity between the recording ultrasonic and reconstructing light wavelength. Reducing the lifesize ultrasonic hologram in the size by the wavelength ratio and, then, viewing it through a high power microscope, the ultrasonically illuminated object can be reconstructed but the 3-D is lost. Under these circumstances the object appears namely to be far away and the viewing aperture is too small for a sideview.

An ultrasonic hologram, however, may be considered as a large scale mask of an optical transform whose diffraction pattern is the original object. C. A. Taylor and H. Lipson demonstrated (1964) that with an optical diffractometer the diffraction pattern of a large scale grid pattern can not only be produced but also reconstructed. So the reconstruction of ultrasonic holograms may be performed on this basis. In Fig. 3, the crystal structure of a casting is visualized by this method. The reconstructed picture, however, looks quite different from that we expect to see on the basis of our optical experiences. This is because interfaces and inner structural irregularities of the piece are sometimes smaller than the ultrasonic wavelength used to record the hologram. Consequently spherical details may appear as a point "highlight" while cylindrical structures appear as line "highlights". Therefore, optically reconstructed ultrasonic holograms *always* require appropriate interpretation, which can be made easier by using the peculiar phenomenon called "colourplastic" described by Rosch. The principle of this phenomenon is that colour spots with sharp boundaries lying in one plane may appear if they would be in different layers. Since in pseudocolour technique the choice of the colour relation is up to us, there is a possibility to give a pseudostereoscopic appearance to the reconstructed ultrasonic hologram.

Fig. 4 is the pseudocolour version of the reconstruction of Fig. 3. Knowing that the casting had parallel surfaces, the colour change can be attributed to different acoustic impedances and so to different crystal structures along the crack.

Although in ultrasonic holography light reconstruction techniques can give valuable information despite the wavelength discrepancy, we are convinced that to overcome all the problems issuing from this discrepancy and to make profit at the same time from all the benefits of ultrasonic holography, we have to change our recording technique principally. One way would be to exploit the possibility of assembling a 3-D image volume from displays issuing from scanning type acoustical-to-optical converters.

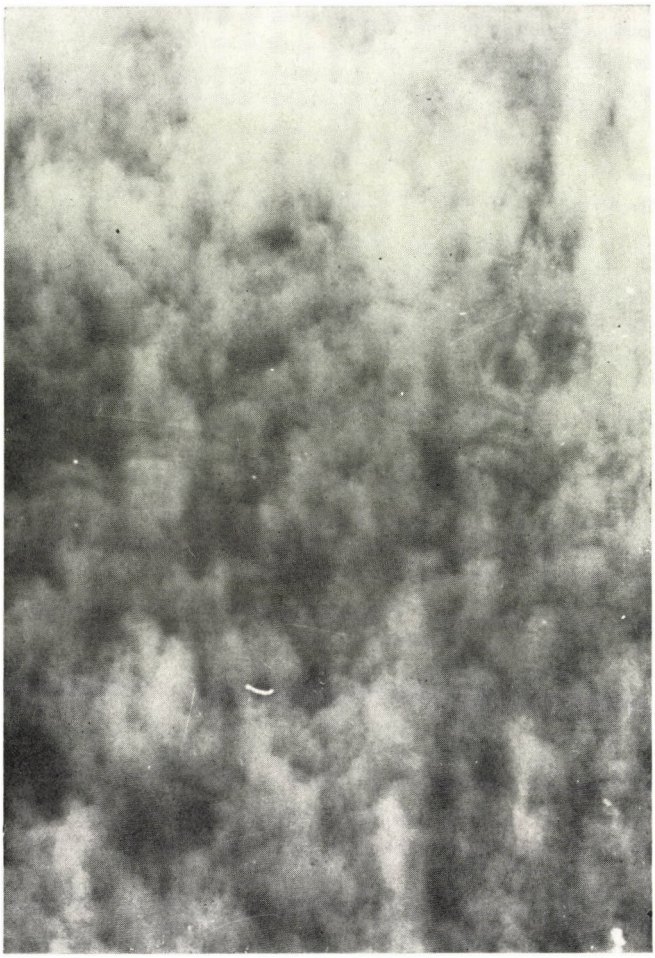

Fig. 3. Crystal structure of a casting visualized by ultrasonic holography

It is well known that on a single holographic recording medium, many separately reconstructable object wave fronts can be recorded. At the same time, the so-called ultrasonic B scan gives us a two-dimensional cross-section picture in the plane of the scan. The information stored in a series of such B scans, when related properly to each other, contains phase-bound data, i.e., three-dimensional information. If these series of ultrasonic B scans are then recorded in one holographic plate by a laser, the resulting multiplex hologram can be regarded as a quasi ultrasonic hologram of the ultrasonically scanned scene, i.e. of the eye or the orbit or both.

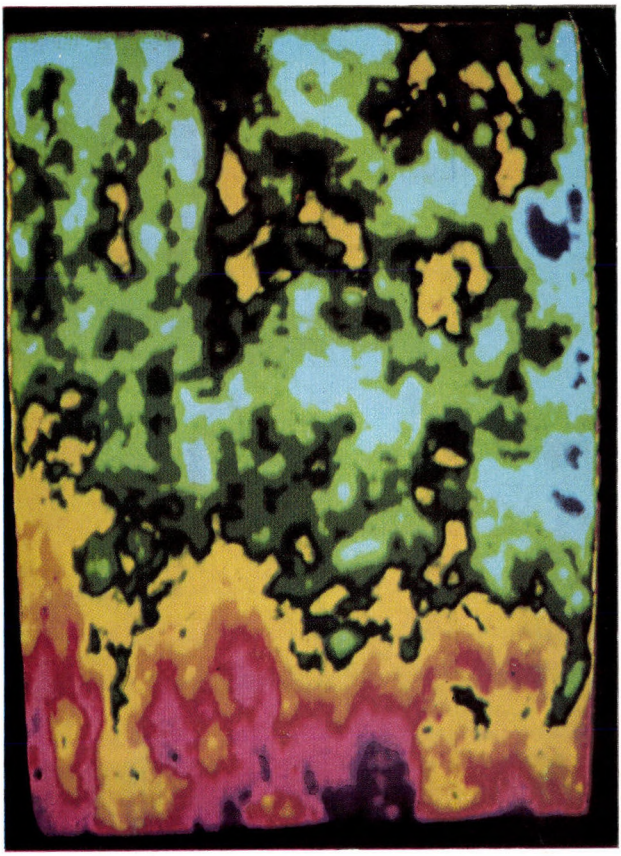

Fig. 4. The pseudo-colour version of Fig. 3 reconstruction

If the geometry of the system is arranged so that at any given viewing angle at least two reconstructed waves enter the observer's eye, a continuous 3-D view of the scene can be seen.

An intraocular foreign body, tumour, etc., can be seen as it is situated in space, and by focusing a measuring microscope on it from different directions, its exact location can be determined.

The reconstructed picture appears to be self-luminous and since in this case not the wave front of the information bearing ultrasonic wave front is reconstructed, but only the recorded B scan sequence, there are no problems issuing from wavelength discrepancies.

In Fig. 5, reconstructed pictures of such an ultrasonic quasi hologram are shown from different viewing angles and focused on different layers. The scene is showing if we would look in the patient's head from its top. So we see the cross section of the right eye, nose bone and left eye. The right eye orbit (black triangle)

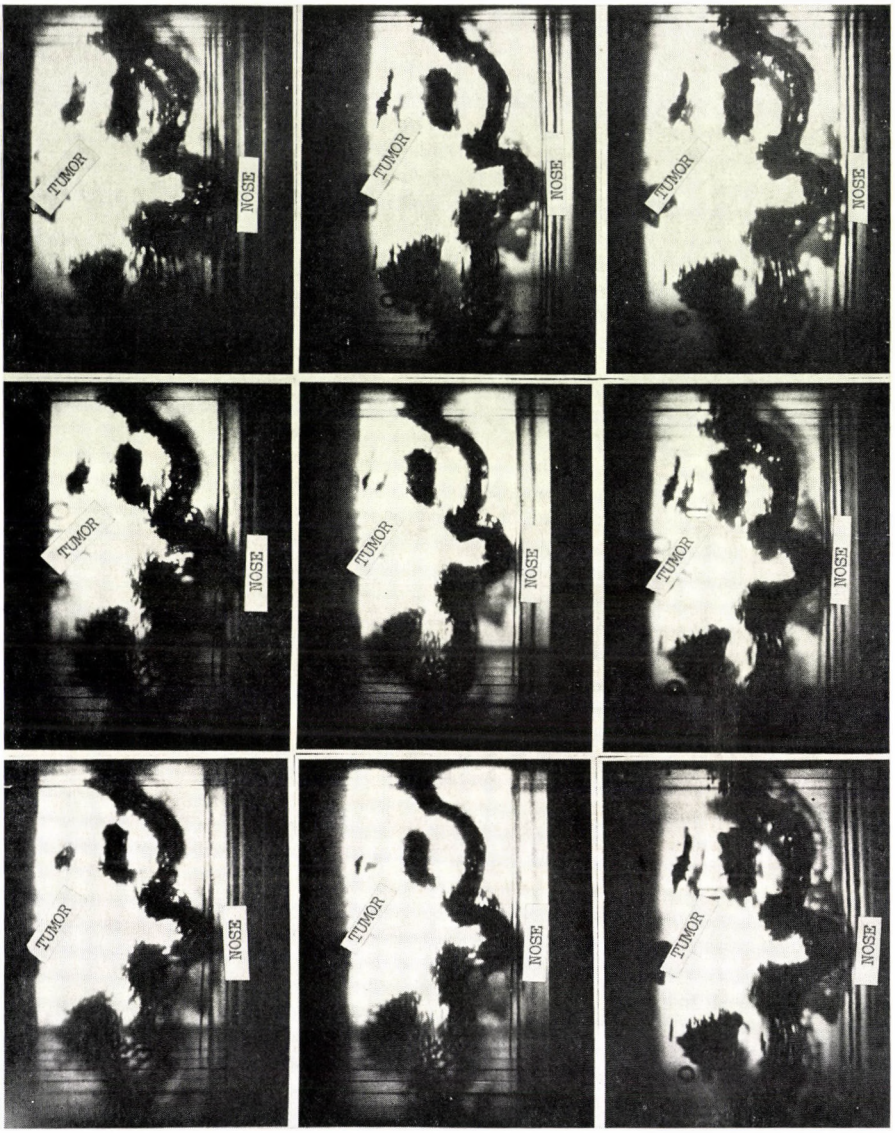

Fig. 5. Reconstructed pictures of an ultrasonic quasi-hologram, showing different viewing angles and focused on different layers

is normal, because at the given setting of the equipment ultrasonic energy is diffusely reflected from the whole orbit. The left orbit, however, has a tumour which absorbs a great deal of the ultrasonic energy and, therefore, we get reflections only from its boundary. Moving our head, the situation of the tumour in space can be perceived and measured.

Bioholography

It is nearly self-evident, when speaking about non-electromagnetic holograms, nobody thinks on information carriers other than some sort of mechanical waves. There is, however, information which is carried and processed by means other than electromagnetic or mechanical waves, e.g. information carried by stimuli in living systems.

The accuracy with which the spatial senses and the brain mechanism associated with them succeed in providing a veridical account of the environment is such that the extent of agreement between the physical world and our experience of it is seldom brought into question. The eye locates information carried by electromagnetic waves, the ear as information receptor serves to pick up information propagated from distant sources on sound, i.e. on mechanical waves, and, in certain cases, information may be submitted by tactual stimuli. Accordingly, the sensory receptors embodied within the retina, the basilar membrane and tactile end-organs are actuated by stimuli of quite different kind with widely differing ranges of *energy sensitivity*. Nevertheless, apparent congruence between these types of information is achieved as a result of a series of extremely complex information processing mechanisms performed simultaneously by the peripheral and central nervous system.

It is well known that information is always combined to one of the components of the information bearing wave, either to its frequency or to its amplitude or to its phase or to all of these three. That means that the biological input could be described exhaustively in the terms of frequency, amplitude and phase. However, all known macroscopic energy receptors, organic and inorganic ones, are *insensitive* to the phase of the information bearing waves. Yet phase is processed in the central nervous system, e.g., animals have 3-D perception which always refers to processed phase information.

At the present stage of our knowledge, the *simultaneous* processing of phase and amplitude bound information can only be achieved by using principles related to holography. Therefore, we suggested several years ago (Greguss, 1967) that biological information processing mechanism should be treated on holographic basis. Evidence that holographic principle may play indeed an important role in biological information processing found support when we predicted, and E. S. Ayrapetyans et al. (1969) have shown, that when a bat emits an ultrasonic pulse of a certain frequency, simultaneously a second brain area, where the echo (target information) is received and processed, is stimulated. The stimulus sent by the programming brain area can be looked upon as the coherent background needed for holorographic information processing. That this is really the case, is backed by the findings that when background noise increases, the intensity of the stimulus informing the receiving brain area of the ultrasonic impulse emission *increases* too, without increasing the intensity of the emitted ultrasonic pulses. This is, however, in good agreement with the basic holographic principle; the signal/noise ratio can be improved by increasing the amplitude of the reference background.

Biological information processing mechanism based on a holographic principle may be involved also in cases when the information carrier wave is independent of the processor, e.g. in visual perception when light waves are carrying the information.

Without going into details of this problem we wish to point out that a living organism as an information processing unit can always be treated as an optical system for which the Huygens principle deduced from the Maxwell equations is valid, i.e. the biomechanism of the information processing can be described principally by the same mathematical methods as it is customary in electron optics and in communication theory. This, however, refers to a neural network which is capable to perform Fourier operations. Our belief that such a neural network exists is based on the recent findings of Szentágothay who pointed out that the cerebellar cortex shows a remarkable regularity, similar to that of a crystalline structure (Szentágothay, 1968). He also drew attention to the very striking phenomenon that the ratio between the climbing fibres and the Purkinje cells is 1 : 1, and that the climbing fibres can be regarded as units reading out the background excitation or inhibition level of the Purkinje cells which were excited by the input of the moss fibres.

The described findings refer, however, to a structure similar to hibrid junctions used in Butler matrices which have been applied by L. Rudolph (Rudolph, Blackwell, 1968) to data processing problems making it possible to perform Fourier transforms with microwaves in nanosecond rates. If we now consider that microwaves propagate with a velocity in the order of 10^{10} cm/s and, to perform Fourier transform, this network needs 10^{-9} seconds, and assuming that the neural network uses similar techniques, and also knowing that the propagation velocity of a stimulus is between $10^{2}-10^{4}$ cm/sec, we get $10^{-1}-1^{-3}$ sec values which is in good agreement with the neural activity rates found experimentally.

The bioholographic information processing model is not only attractive because according to it input information is distribution over the entire recording media, and recall literally implies a reconstruction process (the assembly of dismembered mnemic events), but because it gives answer to the question: what is the material realization when the information pattern is stored? In collaboration with J. Ladik (Ladik, Greguss, 1971) we found that memory mechanism which is based on structural changes in DNA during the information pattern recording process provides a good interpretation of learning, when linked up with this model.

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Quantum Chemical Study of Glucose, G-1-P and G-6-P Molecules as the Effectors of an Enzyme*

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Interaction at large distance of the substrate and enzyme molecules is the initial step of the enzyme action. Theoretical model has been proposed to deal with such systems by the aid of the quantum mechanical perturbation theory. The enzyme glycogen phosphorylase, one of its substrate — glucose-1-phosphate — and a competitive inhibitor — glucose — are used as a model system. It is investigated how important informations can be obtained on the electronic structure of the effectors. Low lying excited levels and wave functions play important part in such discussions. Calculation has been made by the extended Hückel method.

Introduction

The numerical problem connected with the quantum chemical study of the whole enzyme molecule is so enormous that any attempt to make such an investigation is out of question today. Only in such cases is it possible to take the enzyme into account in the calculations when the amino acid sequence of the active site of the enzyme and the biochemical mechanism of the substrate binding are well known. If the atomic structure of the active site of the enzyme has been determined, a model complex of the active site and the substrate molecule may be constructed, and investigations on the quantum chemical properties of this complex may give an insight into interesting features of the enzyme action. Such investigations have been carried out e.g. on the acetylcholinesterase and in the case of the oritidine-5'-phosphate pyrophosphorylase (Hegyháti, Farkas, 1969; Kaneti et al., 1970).

There are very few informations on the above-mentioned properties of the glycogen phosphorylase just as is the case with most of the enzymes (Campbell, Dickens, 1970). Therefore, we have chosen a new approach to the problem, using quantum mechanical analysis of the substrate and some other effector molecules of the enzyme.

The glycogen phosphorylase enzyme catalyzes the reversible reaction:

$$\alpha\text{-D-glucose-1-P} + \text{glycogen}_{(n)} \rightleftharpoons P_i + \text{glycogen}_{(n+1)} \qquad (1)$$

where *-n-* represents the number of glycosyl residues in the polysaccharide, α-D-glucose-1-P is one of the substrates of the enzyme and P_i is the inorganic

* Abbrevations: G-1-P = glucose-1-phosphate; G-6-P = glucose-6-phosphate; UDPG = uridinediphosphoglucose

phosphate. There are some other molecules, – different effectors, – which also can be bound to the active site of the enzyme, e.g. glucose and UDPG. The lower arrow in Euqation (1) is valid under physiological conditions.

The G-6-P molecule, having a structure similar to that of the G-1-P, may serve as a control molecule in our investigations, because there is no interaction between the substrate binding site of glycogen phosphorylase and the G-6-P molecule.

The (1) reaction is pH dependent and its optimum lies at pH $\approx$ 7; therefore, we have regarded the G-1-P and the G-6-P molecules as bivalent anions (Pullman, Pullman, 1963).

A quantum mechanical model of the enzyme substrate interaction

From the quantum mechanical point of view, we may consider the substrate molecule as a physical system, and the effect of the enzyme molecule on the substrate as an external field. Let us imagine that the enzyme and the substrate molecules are apart and the mutual perturbation just begins. Let us further assume that these perturbations are small. Perturbation theory is designed to deal systematically with the effects of such small perturbations on physical systems, when the effects of the perturbations are too difficult to be calculated exactly, and the properties of the unperturbed system are known.

First, let us consider that U^{E} is the unknown weak perturbing potential field of the enzyme. Supposedly we have our substrate molecule as an unperturbed system defined by its ground and excited state wave functions. Thus we can describe the wave function of the perturbed substrate molecule using the results of the Rayleigh-Schrödinger perturbation theory (McWeeny, Sutcliffe, 1969):

$$\Psi_S = \Psi_S^{(0)} + \sum_i \Psi_S^{(i)} \frac{\langle \Psi_S^{(i)} \mid U^E \mid \Psi_S^{(0)} \rangle}{E_0 - E_i}$$

where $\Psi_S^{(0)}$ is the wave function of the ground state of the substrate, and $\Psi_S^{(i)}$ are those of the excited states. The sum goes over the excited states, enumerated by the integer i. The above equation shows that those excited states are mostly contributing to the perturbed wave function – Ψ_S –, which are connected to the ground state by large matrix elements, – $\langle \Psi_S^{(i)} \mid U^E \mid \Psi_S^{(0)} \rangle$ – and smallest $E_0 - E_i$ excitation energies. The value of the excitation energies is smallest in the case of the low-lying excited states. So the effect of the enzyme may be considered as a perturbation on the molecule. The first excited and the other low-lying excited states play a dominant part in the response of the molecule in ground state. From the investigation of the quantum mechanical properties of these low-lying excited states of the molecule we can obtain some informations on the perturbed state of molecule: How does the molecule behave under the influence of the enzyme when they are just approaching each other?

The matrix elements, in which the U^E potential field plays a part, are unknown. However, even from these unknown matrix elements and from the above discussed behaviour of the perturbed molecule some further informations can be obtained. As we have mentioned above the perturbing potential and the wave functions in the matrix elements compete to make the matrix elements large. So these matrix elements together with the wave functions of the ground and excited states of the substrate may hint at the required symmetry and the possible electronic structure of the active site of the enzyme.

Calculating the ground and the excited state properties of the molecules examined, we have used the semi-empirical extended Hückel method. This method takes into account all the valence electrons from the atoms in the treated molecule. A short description of the extended Hückel method, as applied here, can be found in the Appendix.

Table 1

Cartesian coordinates of atoms in G-1-P molecular ion

Atom	X	Y	Z
C_1	1.494255	0.000000	−0.372560
C_2	0.000000	0.000000	0.000000
C_3	0.000000	0.000000	1.560000
C_4	0.817730	−1.138584	2.112280
C_5	2.222212	−1.194925	1.483109
C_6	2.997308	−2.430612	1.944848
O_7	2.109536	−1.194168	0.087651
O_8	2.164678	1.070633	0.010425
P_9	2.745687	1.755583	−1.215819
O_{10}	3.678821	0.803357	−1.933623
O_{11}	3.520553	2.990897	−0.786705
O_{12}	1.623761	2.166219	−2.146074
O_{13}	0.864661	−1.227395	3.508672
O_{14}	−1.400816	−0.147232	1.859393
O_{15}	−0.488612	1.240415	−0.459051
O_{16}	2.297321	−3.207181	2.750676
H_1	2.834465	−3.957055	3.001874
H_2	2.746912	−0.306586	1.802396
H_3	1.378711	−0.496432	3.848350
H_4	0.414153	0.930203	1.920001
H_5	0.078898	1.936897	−0.132278
H_6	1.597689	−0.062162	−1.445797
H_7	3.985237	−2.419665	1.508641
H_8	3.079145	−2.422631	3.021713
H_9	0.247198	−1.999671	1.796966
H_{10}	−1.713688	−0.967714	1.481429
H_{11}	−0.509117	−0.881816	−0.360001

Electronic structure of glucose, G-1-P and G-6-P molecules

Fig. 1 indicates the numbering of the atoms in the G-1-P molecule. The cartesian coordinates of the atoms in the secondary anionic form of the molecule, calculated according to the measured bond distances and bond angles of the α-D-glucose with the C–O–P bond angle of 109°, are shown in Table 1 (Sutton,

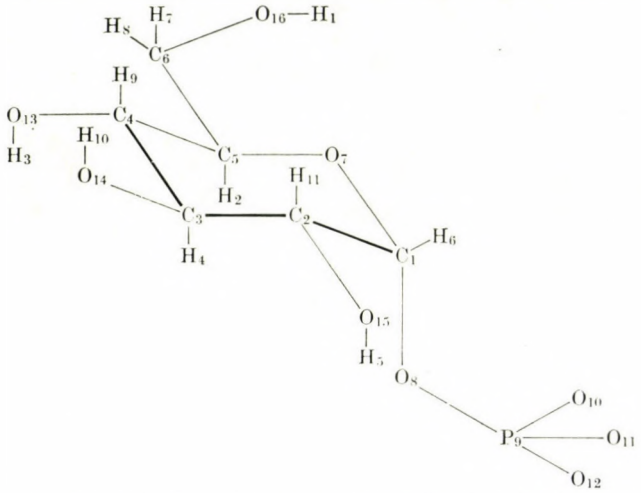

Fig. 1. Numbering of the atoms in the G-1-P molecular ion

Table 2

Charges on the atoms of the G-1-P molecular ion

Atom	Ground state		First excited state	
	Total charge	Net charge	Total charge	Net charge
C_1	2.9060	1.0940	3.1010	0.8990
C_2	3.5091	0.4909	3.6351	0.3649
C_3	3.4798	0.5202	3.5717	0.4283
C_4	3.4950	0.5050	3.6023	0.3977
C_5	3.4922	0.5078	3.6822	0.3178
C_6	4.1868	−0.1868	3.4945	0.5055
O_7	7.2209	−1.2209	7.3059	−1.3059
O_8	7.2692	−1.2692	7.2710	−1.2710
P_9	1.1474	3.8526	1.1512	3.8488
O_{10}	7.7078	−1.7078	7.7080	−1.7080
O_{11}	7.7095	−1.7095	7.7095	−1.7095
O_{12}	7.7067	−1.7067	7.7071	−1.7071
O_{13}	7.2286	−1.2286	7.2607	−1.2607
O_{14}	7.2558	−1.2558	7.2720	−1.2720
O_{15}	7.2364	−1.2364	7.2664	−1.2664
O_{16}	7.1139	−1.1139	7.0064	−1.0064

1958). The charges on the second and third row atoms and the bond orders, computed according to the population analysis method developed by Mulliken, are shown in Table 2 and Table 3 (Mulliken, 1955).

Fig. 2 indicates the numbering of the second row atoms in glucose molecule. The computed atomic charges and bond orders for the glucose molecule are shown in Table 4 and Table 5.

Table 3

Bond orders in the G-1-P molecular ion

Bond	Bond orders	
	Ground state	First excited state
C_1-C_2	0.7707	0.8624
C_2-C_3	0.7638	0.7104
C_3-C_4	0.7927	0.8314
C_4-C_5	0.7564	0.8144
C_5-C_6	0.7714	0.7665
C_1-O_7	0.4431	0.2848
C_5-O_7	0.4456	0.2358
C_1-O_8	0.5313	0.5169
C_2-O_{15}	0.4336	0.3403
C_3-O_{14}	0.4055	0.3541
C_4-O_{13}	0.4350	0.3345
C_6-O_{16}	0.3203	0.4412
O_8-P_9	0.4095	0.4078
P_9-O_{10}	0.4260	0.4254
P_9-O_{11}	0.4161	0.4156
P_9-O_{12}	0.4256	0.4246

Table 4

Charges on the atoms of the glucose molecule

Atom	Ground state		First excited state	
	Total charge	Net charge	Total charge	Net charge
C_1	2.8819	11.181	3.0869	0.9131
C_2	3.5090	0.4910	3.6356	0.3644
C_3	3.4797	0.5203	3.5683	0.4317
C_4	3.4951	0.5059	3.5921	0.4079
C_5	3.4924	0.5076	3.6733	0.3267
C_6	4.1869	−0.1869	3.4935	0.5056
O_7	7.2214	−1.2214	7.3043	−1.3043
O_8	7.1840	−1.1840	7.1898	−1.1898
O_9	7.2286	−1.2286	7.2576	−1.2576
O_{10}	7.2558	−1.2558	7.2715	−1.2715
O_{11}	7.2341	−1.2341	7.2657	−1.2657
O_{12}	7.1140	−1.1140	7.0086	−1.0086

Table 5

Bond orders in the glucose molecule

Bond	Bond orders	
	Ground state	First excited state
C_1-C_2	0.7718	0.7896
C_2-C_3	0.7638	0.8372
C_3-C_4	0.7926	0.7897
C_4-C_5	0.7565	0.7531
C_5-C_6	0.7714	0.8216
C_1-O_7	0.4441	0.4373
C_5-O_7	0.4455	0.5499
C_1-O_8	0.5278	0.5385
C_2-O_{11}	0.4340	0.4489
C_3-O_{10}	0.4056	0.4220
C_4-O_9	0.4350	0.4789
C_6-O_{12}	0.3204	0.3184

Table 6

Charges on the atoms of the G-6-P molecular ion

Atom	Ground state		First excited state	
	Total charge	Net charge	Total charge	Net charge
C_1	2.8819	1.1181	2.8890	1.1110
C_2	3.5090	0.4910	3.3220	0.6780
C_3	3.4803	0.5197	3.3634	0.6366
C_4	3.4943	0.5057	3.5898	0.4102
C_5	3.4960	0.5040	3.6665	0.3335
C_6	3.6329	0.3671	3.6094	0.3906
O_7	7.2161	−1.2161	7.2996	−1.2996
O_8	7.1840	−1.1840	7.1759	−1.1759
O_9	7.2274	−1.2274	7.2505	−1.2505
O_{10}	7.2558	−1.2558	7.2507	−1.2507
O_{11}	7.2342	−1.2342	7.2468	−1.2468
O_{12}	7.2869	−1.2869	7.2855	−1.2855
P_{13}	1.1452	3.8548	1.1452	3.8548
O_{14}	7.7101	−1.7101	7.7100	−1.7100
O_{15}	7.7069	−1.7069	7.7069	−1.7069
O_{16}	7.7067	−1.7067	7.7067	−1.7067

The numbering of the second and third row atoms are illustrated also for the G-6-P ion in Fig. 3. The atomic charges and bond orders for this case are shown in Table 6 and Table 7. These charges and bond order values for the molecular ions have been obtained by considering the valence electrons of the secondary anionic form of these molecules.

Comparing the charge distribution of G-1-P, glucose and G-6-P molecules in their ground and first excited states we have observed considerable change on the C_6 carbon atom in the case of G-1-P and glucose molecules. A similar com-

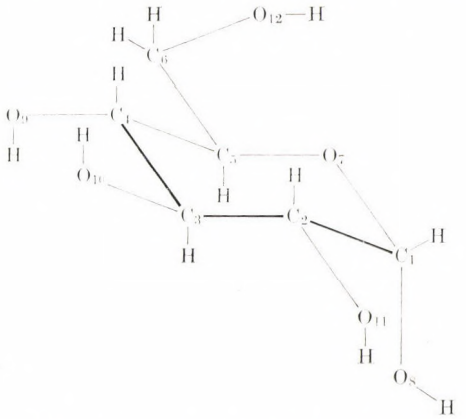

Fig. 2. Numbering of the second row atoms in the glucose molecule

Table 7

Bond orders in the G-6-P molecular ion

Bond	Bond orders	
	Ground state	First excited state
C_1-C_2	0.7719	0.8048
C_2-C_3	0.7639	0.9474
C_3-C_4	0.7931	0.7873
C_4-C_5	0.7642	0.7563
C_5-C_6	0.7520	0.7559
C_1-O_7	0.4442	0.4182
C_5-O_7	0.4548	0.5637
C_1-O_8	0.5277	0.5348
C_2-O_{11}	0.4340	0.4457
C_3-O_{10}	0.4055	0.4188
C_4-O_9	0.4361	0.4772
C_6-O_{12}	0.4815	0.4817
$O_{12}-P_{13}$	0.4188	0.4187
$P_{13}-O_{14}$	0.4313	0.4313
$P_{13}-O_{15}$	0.4188	0.4188
$P_{13}-O_{16}$	0.4194	0.4194

parison of the bond orders in the G-1-P molecule shows that the C_1-O_7 and C_5-O_7 bonds are weaker in the excited state. However, there is a gain in strength at the C_5-O_7 bonds for the glucose and G-6-P molecules.

The observed charge distributions and bond orders of the other low-lying excited states were similar to the corresponding properties of the first excited state in all cases of the examined molecules.

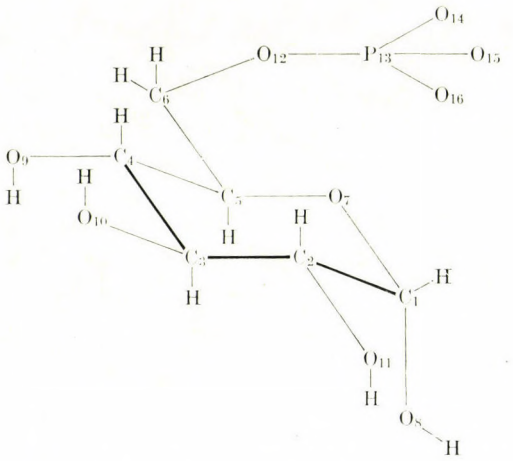

Fig. 3. Numbering of the second and third row atoms in the G-6-P molecular ion

Discussion

The quantum mechanical study of G-1-P – a substrate molecule of the glycogen phosphorylase enzyme – shows that a perturbation having large matrix elements between the ground and the low-lying excited states may considerably weaken the C_1-O_7 and C_5-O_7 bonds.

Favourable place for the polarization attack of the enzyme may be around the carbon atom with index 6, where the change in the atom charge is the greatest one when going from the ground state to the low-lying excited states of the investigated substrate and inhibitor molecules. The location of the polarization effect of the glycogen phosphorylase enzyme at the C_6 atom is supported by the results obtained from the study of the G-6-P molecule. In the case of G-6-P, there is no atom-charge change on the C_6 atom and, really, there is no specific interaction between the G-6-P molecule and the substrate binding site of the glycogen phosphorylase enzyme.

Comparison of the bond order values of G-1-P, glucose and G-6-P molecules shows that the weakening of the C_1-O_7 and C_5-O_7 bonds is the specific property of the G-1-P molecule. The G-1-P molecule may be recognized by the enzyme through this property.

Appendix

The extended Hückel method used in the present paper was originally described in the paper by Hoffmann (1963). The method is based on the Hückel method with inclusion of overlap. In the calculations of G-1-P and G-6-P molecules we have used a basis set consisting of $m = 11$ hydrogen Slater orbitals, $n = 15$ $2s$ and $3n = 45$ $2p$ Slater orbitals for carbon and oxygen atoms and $n' = 1$ $3s$ and $3n' = 3$ $3p$ Slater orbitals for phosphorus atom. In the case of the glucose molecule the basis set used consisted of $m = 12$ hydrogen Slater orbitals, $n = 12$ $2s$ and $3n = 36$ $2p$ Slater orbitals for oxygen and carbon atoms. The calculated exponents of the Slater orbitals and the ionization potential values used in the calculations approximating the diagonal matrix elements in the method are listed in Table 8. The particular ionization potential values used for C, O and H atoms

Table 8

Ionization potential and Slater exponent values used in the calculations

Atom	H_{ii} ($1s$, $2s$ or $3s$)	H_{ii} ($2p$ or $3p$)	Slater exponent
H	−13.60 eV	—	1.000
C	−21.01 eV	−11.27 eV	1.625
O	−36.07 eV	−18.53 eV	2.275
P	−18.36 eV	−9.79 eV	1.600

have been chosen from the paper by Hinze and Jaffé (1962) and, for the P atom, from the paper Issleib and Gründler (1966). The off-diagonal matrix elements have been estimated by the Mulliken approximation, i.e. $H_{ij} = C S_{ij} (H_{ij} + H_{jj})$ where C in our calculations was taken for 1.75 (Dewar, 1969).

The study of overlap populations, computed from the LCAO coefficients, gave the distribution of electronic population among the atoms in the molecules.

The CDC 3300 computer of the Hungarian Academy of Sciences has been used for the numerical calculations.

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Comparison of the Quantum Chemical Models of Some Biological Important Phosphates

(Short Communication)

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(Received January 15, 1972)

Quantum chemical calculations have been made for various organic phosphates by Grabe (1958) and by A. and B. Pullman (1960). The method used in the previous calculations was the application of the semi-empirical Hückel molecular orbital method. Results of these calculations show that a particular pi electron arrangement is characteristic of the energy-rich phosphates, an arrangement generally considered unstable in the pi electron approximation. This type of molecules always presents a chain of at least three neighbouring atoms, the net charge of which is positive. This property can be observed on five or, in some cases, on six neighbouring atoms in certain phosphates. Such energy-rich organic phosphates are the creatine phosphate, acetyl phosphate and phosphoenolpyruvate. This property cannot be found in phosphates not considered to be of energy-rich character and having only the maximal number of two neighbouring atoms with positive net charges, as is the case with glucose-6-phosphate.

The general opinion is that the problem of the virtual lack of configurational instability of these molecules may be overcome by consideration of the sigma electronic structure of the molecule.

On the basis of these considerations we have examined the electronic structures of some energy-rich and energy-poor organic phosphates with the extended Hückel method, which enables one to deal with sigma and pi electrons on an equal footing.

We have calculated the charge distribution of the above-mentioned energy-rich and energy-poor phosphates and of the glucose-1-phosphate molecule. The calculations were carried out in the framework of the extended Hückel method developed by Hoffmann (1963). Details of the method were described in our previous paper (Gáspár, 1972), where the numerical values for the parameters can be found in Table 8. Values for the nitrogen atom, that we have used, were -26.92 eV as 2s and -14.42 eV as 2p ionization potentials and the value of the calculated Slater exponent was 1.950.

The cartesian coordinates of the atoms were calculated in the investigated molecules by the aid of the bond angles and bond distances tabulated by Sutton, with the C–O–P bond angles of 109° (Sutton, 1958). The phosphoryl residue

was taken into account in the form of a secondary anion

$$\begin{array}{l} O^- \\ | \\ -P-O^- \\ || \\ O \end{array}$$

according to Pullman's description (1963). The distribution of the electronic charges in the phosphate groups and their surroundings, obtained by the population analysis, are shown in Fig. 1 for the molecules in consideration (Mulliken, 1955).

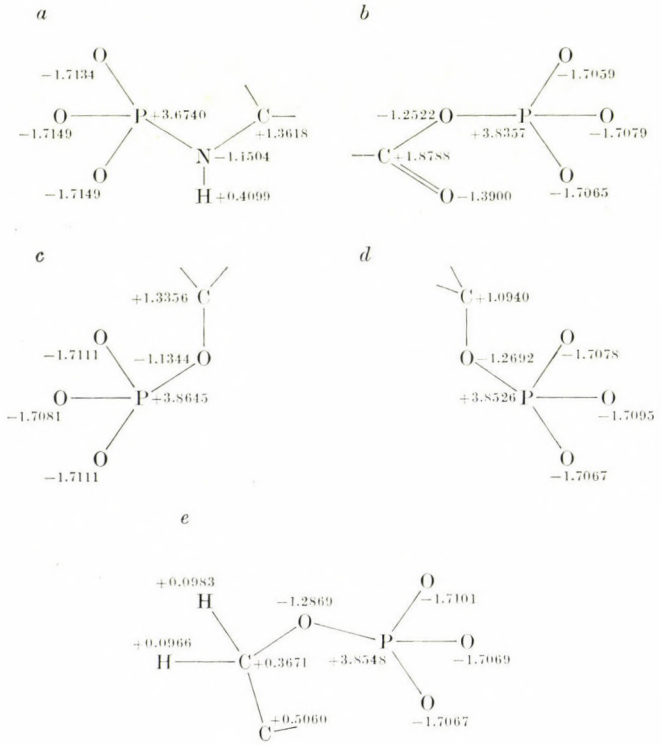

Fig. 1. Distribution of net electronic charges in the phosphate groups and their surroundings. *a*: Creatine phosphate; *b*: Acetyl phosphate; *c*: Phosphoenolpyruvate; *d*: Glucose-1-phosphate; *e*: Glucose-6-phosphate

According to the extended Hückel theory it immediately appears that the net charge is negative on the atom – oxygen or nitrogen – which serves as a bridge between the organic radical and the phosphate group. It seems that the special property which indicates the energy-rich or energy-poor property of the compound investigated is the net charge value of the terminal carbon atom of the organic radical. For the energy-rich compounds we have observed larger net charge

values than for the glucose-1-phosphate, supposedly being an intermediate-energy phosphate and, for the glucose-1-phosphate, a larger value than in the case of the energy-poor glucose-6-phosphate.

The extension of the calculations by simultaneous consideration of the sigma and pi electrons has changed the charge distribution considerably in comparison with that obtained by considering the pi electrons alone.

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On the Electron Donor Property of Veratrine

(Preliminary Communication)

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In the past decade a number of experiments was performed on charge-transfer reactions in model systems of biological interest in order to study the subtlety of biological processes on submolecular level (Sukhorukov et al., 1966; Lybchenko, Sukhorukov, 1969; Grecsiskina, 1969; Lohmann, 1971).

Recently Szent-Györgyi and his coworkers (Szent-Györgyi, 1967, 1969; Kimura, Szent-Györgyi, 1969; McLaughlin, 1968) have carried out investigations on biological tissues and concluded that the electron donor-acceptor interactions may play an important role in living systems.

It is well-known from the physiological experiments of Lorente de No (1947), Fleckenstein (1951) and Bíró (1969) that the activity of nerves, measured by recording the action potential, decreases if the nerves are kept in physiological Ringer solution containing a small amount of veratrine (the stimulus threshold increased and, after a longer incubation time, the nerves lost their ability to conduct excitation). On the other hand, it has been pointed out that the block evoked by veratrine could be temporarily influenced and suspended by dissolving procaine in the Ringer solution. Finally, in order to interpret the physiological phenomena, Ernst (1966, 1968) assumed veratrine and procaine to act as electron donor agents in biological systems, and in the nerve tissue in particular.

The presence of a charge-transfer interaction should in general be studied by means of the paramagnetism measured under favourable conditions. Therefore, electron paramagnetic resonance (epr) experiments were performed on veratrine-organic acceptor systems in order to modelize the biological effect of veratrine.

In the course of the experiments 10, 50 or 100 mM/l of veratrine hydrochloride and of tetrachloro-p-benzoquinone (chloranil), p-benzoquinone or tetracyanoethylene were dissolved in acetonitril, dioxane or ethylene glycol. They were prepared and mixed in equimolar amounts immediately before the experiments. All chemicals used in the experiments were of analytical grade and were not further purified.

The electron paramagnetic resonance spectra were taken at room temperature using a Carl Zeiss ER 9 spectrometer operating at 9370 Mc/s. All spectra of the model systems were recorded at the same power setting, modulation amplitude was chosen to be 0.4 G. The first derivative of the absorption curve was recorded.

We have found that each of the substances investigated gave electron spin resonance signal under the condition described, a fact indicating that veratrine, as an electron donor, readily gives off one electron to share with another molecule. In the solutions which do not contain veratrine or electron acceptor, no epr signal was observed. Fig. 1a shows the epr spectrum of a 1 : 1 veratrine-chloranil sample

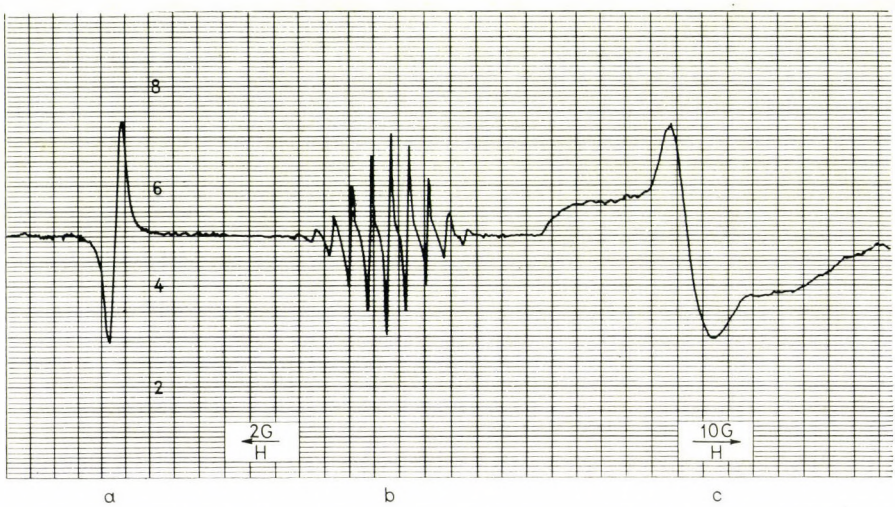

Fig. 1 (a) The electron spin resonance spectrum of the 1 : 1 veratrine-chloranil sample dissolved in acetonitril, (b) spectrum of 1 : 1 veratrine-tetracyanoethylene in ethylene glycol, (c) 1 : 1 veratrine-chloranil sample in solid phase

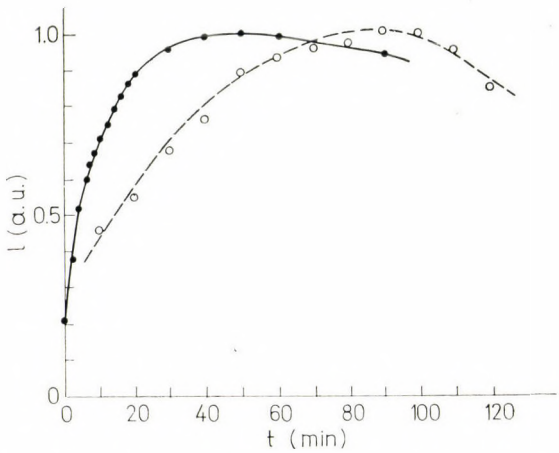

Fig. 2. Kinetic curves of veratrine-organic acceptor systems. The intensity of the free radical concentration measured in arbitrary units is plotted against time; (●–●–●) 1 : 1 veratrine-chloranil sample in dioxane; (○–○–○) 1 : 1 veratrine-tetracyanoethylene sample dissolved in ethylene glycol. The values represent the mean of several determinations

(50 mM) dissolved in acetonitril. Fig. 1b demonstrates the spectrum of a 1 : 1 veratrine-tetracyanoethylene sample (50 mM) dissolved in ethylene glycol. Fig. 1c represents the epr spectrum of a mixture of veratrine and tetrachloro-p-quinone in solid phase in molar concentration. The sample was prepared by smearing the components mechanically. The spectra obtained in the experiments are in good agreement with the epr spectra of the negative ion radicals of the acceptors as regards the g-factor, line width and hyperfine splitting constants described in the literature, except for the spectrum indicated in Fig. 1c.

It should be noted that the intensity of the paramagnetic signals showed the kinetics described by Eastman et al. in 1962; the initial increase was followed by approach to a slow asymptotic rate before all the paramagnetic species have disappeared (Fig. 2). We have not yet analyzed the dependence of the rate on the initial concentration and time. This kind of kinetic behaviour seems to be characteristic of the radical reactions investigated in relation with veratrine-organic acceptor systems.

The experiments performed by us on model systems seem to support the assumption that veratrine may act as an electron donor, a finding being in accordance with the results of physiological experiments.

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Book Review

Biokybernetik. Band III. (Papers of the Second International Symposium "Biocybernetics" in Leipzig.) Edited by Hans Drischel and Norbert Tiedt, Karl Marx University Leipzig. VEB Gustav Fischer Verlag, Jena. 1971; 315 pp., 223 figures and 7 tables.

This book consists of the 52 papers presented on the Second International Symposium "Biocybernetics" in Leipzig. (The first Symposium on "Biocybernetics" was held in 1967 and its subject was edited in the two previous volumes of "Biokybernetik". This symposium covered a large scale of experimental biocybernetics and of theoretical-methodological topics). In comparison with the first symposium on biocybernetics, the subject of the second conference was concentrated on a few special topics of biocybernetics, making an intensified study of problems possible.

I. *General and theoretical aspects* (17 papers). Mainly the problems of modelling are reported, a few papers concern with the connection between learning process and information theory; some mathematical methods, the behaviour of dynamic systems, periodic and circular processes. An interesting theoretical paper deals with the possibility of using computers in medicine to detect disease and to decide that which category of disease is implicated.

II. *Motor system* (12 papers). Mostly the muscle spindle function and muscular contraction processes are analysed and modelled besides the study of different receptor functions and of reflex systems.

III. *Visual system* (19 papers). Processing of visual information, binocular and colour vision, the rate of visual perception, accommodation, recognition of visual patterns electrophysiological studies (involving an interesting work about the interaction between visually and auditorily evoked DC-potentials), and pupillary mechanisms are the main topics of this chapter.

IV. *Heart, circulation and other homeostatic mechanisms* (4 papers). One of the papers presents experimental studies of autoregulation of flow on isolated hindlimb and head preparation under the effect of rectangular pressure steps, two papers deal with blood pressure regulatory mechanisms, the last one is the summary of a mathematical model for the six chambered heart.

The papers are mostly in English (38 out of 52) the remainder 14 papers are in German. A less part of the lectures is summarized only, but each of them is supplied with the most important references. Content of the book is presented in English as well as in German.

Authors and their addresses are listed at the end of the volume. A supplement enumerates the titles of 37 papers which were presented on the Symposium but not in the book.

Though the presentation of the (very likely interesting) spoken discussion which characterize every symposia is completely missing, the reader has got a very valuable and useful survey of the worldwide effort of experts to find an exact description of living systems.

T. LAKATOS

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W DE G

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ANNOUNCEMENT OF AN IAEA SYMPOSIUM

Title: Radioimmunoassay and Related Procedures in Clinical Medicine and Research

Date: 4—8 June 1973 (subject to confirmation)

Location: Istanbul, Turkey (subject to confirmation)

Organizers: International Atomic Energy Agency
Kärntner Ring 11—13, A—1010 Vienna, Austria

Scientific Secretaries: Dr. E. J. Garcia and Dr. E. H. Belcher Medical Applications Section

The programme of this Symposium, the second to be organized by the International Atomic Energy Agency in the subject field, will cover all aspects of radioimmunoassay and related procedures and their applications. Particular attention will be given to the *in vitro* determination of substances of clinical interest such as hormones, vitamins and drugs. Sections of the programme will be devoted to recently developed techniques for the detection of tumour, viral and bacterial antigens, and to assays employing labelled antibodies and naturally occurring specific receptors.

Further information, participation forms and forms for submission of a paper intended for presentation at the Symposium will be obtainable from national authorities for atomic energy matters. Abstracts of such papers must be submitted to the International Atomic Energy Agency through these authorities.

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Re-examination of the Sequence of the C-terminal Tryptic Fragment from Porcine Adrenocorticotropic Hormone

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(Received July 25, 1972)

The C-terminal tryptic fragment of porcine adrenocorticotropic hormone (residues 22—39) was digested with papain. All the papain fragments were isolated and subjected to sequence analysis. The overlaps observed permit unambiguous alignment of the fragments. The difference between the sequence given in this report and that proposed by Shepherd et al. (1956b) pertains only to the position of the single amide group of the molecule: we found that aspartic acid at position 25 is amidated instead of glutamic acid at position 30.

Introduction

The conversion of porcine adrenocorticotropic hormone (α_p-ACTH) in mild alkaline medium to a more acidic component is well known in the literature (Shepherd et al., 1956a; Dixon, Stack-Dunne, 1955). This change was attributed to deamidation of the glutamine residue at position 30 (Shepherd et al., 1956a). Our studies on the mechanism of alkaline deamidation of different peptides have made the validity of location of this labile amide group questionable, and this prompted us to re-examine the sequence of porcine ACTH in the critical C-terminal region. The results of our analysis have been reported in a preliminary paper (Gráf et al., 1971). According to these studies the structure of α_p-ACTH differs from that described by Shepherd et al. (1956b) in the position of the single amide group. Meanwhile, Riniker et al. (1972) proposed the same revision for the sequence of porcine ACTH. Since their analysis was restricted to a narrower region of the sequence, a detailed publication of our sequence studies on the C-terminal tryptic fragment of α_p-ATCH seems warranted. In this report the sequences of all the fragments obtained by papain cleavage of the C-terminal tryptic fragment of α_p-ACTH are given.

Materials and methods

Porcine ACTH was prepared by the procedure of Birk and Li (1964), which is routinely employed for the isolation of lipotropic hormones in our laboratory (Gráf, Cseh, 1968). The most basic fraction of ACTH was further purified by chromatography on a carboxymethyl cellulose (Whatman CM32) column

equilibrated and eluted with the same buffer: 0.13 M ammonium acetate, pH 6.7. ACTH obtained was identified on the basis of biological effect (Purves, Sirett, 1965), end-group analysis (Gray, 1967), amino acid analysis, gel electrophoresis (Gráf et al., 1971) and mapping of its tryptic digest (Gráf et al., 1970a). The biological activity of our preparation was 91 IU/mg. The presence of deamidated and oxidized artifacts in this preparation was excluded by gel electrophoresis and detection of methionine from the aminopeptidase M digest of the hormone.

ACTH was digested with trypsin in 0.05 M ammonium acetate, pH 7.5, at 37°C for 2 hours with an enzyme to peptide ratio of 1 : 50 (w/w). Papain (Sigma) cleavage on the C-terminal tryptic fragment (CTT) was performed in 0.1 M pyridine acetate, pH 5.5, containing 0.005 M cysteine and 0.005 M ethylene-diamine-tetra-acetate, with an enzyme to peptide ratio of 1 : 50 at 37°C for 3 hours. Two-dimensional paper electrophoresis of the digests was carried out on Whatman 3MM paper, first at pH 5.0 (pyridine – acetic acid – water, 10 : 10 : 1000) and subsequently at pH 2.0 (formic acid – acetic acid – water, 50 : 150 : 800). Maps were developed by dipping the paper into a 1% ninhydrin solution in acetone containing 0.1% cadmium acetate, 5% acetic acid and 5% water. Homogeneous peptides were eluted from the paper and subjected to amino acid and sequence analysis.

Acid hydrolysis was performed with 6 M HCl for 45 hours at 110°C in sealed, evacuated, tubes. Aminopeptidase M (Röhm-Haas) hydrolysis was performed in 0.1 M Tris buffer, pH 7.6, with an enzyme to peptide ratio of about 1 : 1 (w/w) at 37°C for 5 hours. Digestion with carboxypeptidase A (Worthington) was made in 0.05 M $NaHCO_3$ (except for peptide n_{11}, which was digested in 0.05 M NH_4HCO_3, pH 8) with an enzyme to peptide ratio of 1 : 10 (w/w) for 8 hours. Acid and enzymatic hydrolysates of the peptides were analyzed on a JEOL (JLC-5AH) automatic analyzer. Peptides were sequenced by the Edman-dansyl method (Gray, 1967). To distinguish asparagine from aspartic acid and glutamine from glutamic acid, beside the analysis of exopeptidase digests, the critical fragments were run at pH 6.5 and the number of free carboxyl groups was calculated from the relative mobilities (Offord, 1966).

Results and discussion

The single acidic fragment from the tryptic digest of α_p-ACTH was isolated by paper electrophoresis at pH 5. The amino acid compositon of this fragment was found to be: Asp_2, Glu_1, Pro_2, Gly_1, Ala_3, Val_1, Leu_2, Tyr_1, Phe_2. Edman-dansyl procedure gave the following result for its NH_2-terminal sequence: Val-Tyr-Pro-Asx-Gly-Ala-Glx-. Carboxypeptidase A cleaved Phe, Glu, Leu from the fragment in equimolar quantities. These data show that this fragment represents the C-terminal portion (residues 22 – 39) of porcine ACTH (Shepherd et al., 1956b).

Fig. 1 shows the location of the papain-peptides derived from CTT. All the peptides shown on the map were isolated by two-dimensional preparative paper

electrophoresis and subjected to amino acid and sequence analysis (Table 1). The sequence of peptides sufficiently overlap so that their alignment is possible. To determine the position of amide group(s), each glutamic acid and aspartic acid residue in CTT was examined. The neutrality of peptide n_{11} proves the presence of asparagine at position 25 instead of aspartic acid. An additional evidence for this was provided by the digestion of peptide n_{11} with carboxypeptidase A (Table 2). The acidic character of fragment a_{11} indicates that at position 28 a glut-

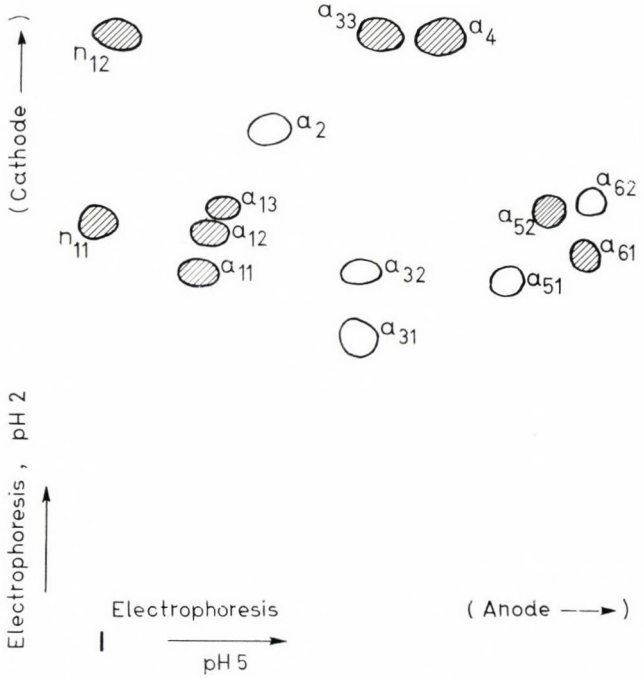

Fig. 1. Peptide map of a papain digest of the C-terminal tryptic fragment from α_p-ACTH made by electrophoreses at pH 5 and 2. Spots of cystine and cysteine (the latter was added to the system) are not indicated on the map. Spots of more intensive ninhydrin color are shaded

amic acid is present. The analysis of the aminopeptidase M hydrolysate of fragment a_{51} supports this suggestion (Table 2). Peptide a_{61} could be almost completely hydrolyzed by aminopeptidase M and the amino acid composition fairly agrees with that of the acid hydrolysate of the same fragment (Table 2). On the basis of this observation it appears that carboxyl side chains of Asp-29, Glu-30 and Glu-33 are free. Glu-30 was also checked by carboxypeptidase A cleavage of fragment a_{31}. Finally, digestion of CTT with carboxypeptidase A results in the release of a glutamic acid (beside Leu und Phe) which is located at position 38. The determination of the relative mobilities of the critical fragments gave a further support for this location of the amide group.

Table 1

Amino acid sequence and composition of papain peptides of the C-terminal tryptic fragment α_p-ACTH

Peptide	Sequence
n_{11}	Val.Tyr.Pro.Asn.Gly
a_{11}	Val.Tyr.Pro.Asn.Gly.Ala.Glu
a_{31}	Val.Tyr.Pro/Asx,Gly,Ala,Glx,Asp/Glu.Leu.Ala
a_{33}	Ala.Glu
a_{62}	Ala.Glu.Asp.Glu
a_{51}	Ala.Glu.Asp.Glu/Leu,Ala/
a_{52}	Asp.Glu/Leu,Ala/
a_{61}	Asp.Glu.Leu.Ala.Glu
a_2	Leu.Ala.Glu
a_4	Glu.Ala
a_{32}	Glu.Ala.Phe/Pro,Leu,Glu/
a_{12}	Ala.Phe.Pro.Leu.Glu
a_{13}	Phe.Pro.Leu.Glu
n_{12}	Phe
CTT	Val.Tyr.Pro.Asn.Gly.Ala.Glu.Asp.Glu.Leu.Ala.Glu.Ala.Phe.Pro.Leu.Glu.Phe
	22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 27 38 39

Table 2

Digestion of papain fragments of α_p-ACTH with carboxypeptidase A and aminopeptidase M

amino acid	Amount in the digest (mole/mole peptide*) obtained with				
	carboxypeptidase A			aminopeptidase M	
	CTT	a_{31}	n_{11}	a_{51}	a_{61}
Asp	—	0.25	—	0.09	0.71
Asn	—	—	0.25	—	—
Glu	0.81	0.48	—	0.35	1.25
Gln	—	—	—	—	—
Gly	—	—	0.35	—	—
Ala	—	0.75	—	0.71	0.77
Leu	0.83	0.76	—	0.12	0.68
Phe	0.77	—	—	—	—

* Amount of peptides was determined by analyzing their acid hydrolysates.

The sequence based on the above data agrees with that proposed by Riniker et al. (1972). The revision of the sequence established by Shepherd et al. (1956b) affects two amino acid residues, i.e. Asp or Asn at position 25 and Gln or Glu at position 30. These residues are outside the active center generally accepted for ACTH (Schwyzer et al., 1971). However, according to our studies the state of amidation of these residues influences biological activity (Gráf et al., 1970b).

The amide group was re-assigned by us to that region of the molecule (residues 25–28) in connection of which a "special problem" of the analysis was mentioned by Shepherd et al. (1956b). According to these authors in the course of Edman-degradation of the C-terminal tryptic fragment ("T_1") after the removal of Val, Tyr and Pro, several end-groups were obtained, which made further analysis impossible. This reminds us of the phenomenon we met at position 25 when analyzing the deamidated derivative of α_p-ACTH (Gráf et al., 1971). The heterogeneity observed by Shepherd et al. (1956b) might also be due to transpeptidation involved in the reaction of deamidation. Sequence 25–28 was finally established by Shepherd et al. (1956b) from data provided by partial acid hydrolysis. This mode of cleavage alone cannot distinguish amidated and free carboxyl side chains. The location of amide at position 30 seems to be well established by Shepherd et al. (1956b). The apparent contradiction between our results and those of Shepherd et al. (1956b) is at present unexplained.

I thank Drs S. Bajusz, G. Cseh and M. Sajgó for many helpful discussions and Dr A. Patthy for the amino acid analyses. For the biological measurements thanks are due to Dr G. Hajós (Gedeon Richter Factory, Budapest).

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The Heterogeneity of Informofer

II. Differential Effect of Actinomycin-D on Nuclear Particles Containing Different Types of dRNA

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(Received March 27, 1972)

By means of fractionated extraction ribonucleoprotein particles containing different dRNA species can be isolated from rat liver cell nucleus. The effect of actinomycin-D was studied on the ribonucleoprotein fractions containing dRNA and extracted at 0.1 and 0.3 M NaCl concentrations during the course of a chase following ^{14}C-orotate administration. In addition, the incorporation of ^{14}C-orotate into these particles was examined on the effect of various actinomycin-D concentrations.

It was found that the amount of nuclear particles extracted at 0.1 M NaCl concentration decreased very rapidly and markedly during the chase, whereas with the particles extracted with 0.3 M NaCl a change of similar character but of considerably smaller extent could be detected. The incorporation of ^{14}C-orotate into nuclear ribonucleoproteins containing dRNA could be demonstrated even after the administration of 4.7 mg/kg actinomycin-D. Also in this case the radioactivity of particles extracted with 0.3 M NaCl was higher. As shown by electron microscopy, the number of interchromatic granules decreased in the nucleus after actinomycin-D administration.

The experiments corroborate our earlier observation that the informofer is heterogeneous: the "0.1 M informofer" forms complexes with mRNA, whereas the "0.3 M informofer" with the dRNA sequences degrading inside the nucleus. The fractionated extraction renders it feasible to prepare ribonucleoprotein fractions rich in mRNA molecules or in dRNA sequences degraded in the nucleus.

In eukaryotes only a part of newly formed giant dRNA molecules is transferred to the cytoplasm after cleavage the rest remains in the nucleus and is rapidly degraded there (Scherrer et al., 1970; Georgiev et al., 1972). The latter fraction is strongly bound to chromatin and it can be extracted only at higher temperature (Arion, Georgiev, 1967) or by increasing the ionic strength of extracting medium (Drews, 1969). We have earlier described that the dRNA fraction that is extracted at 0.3 M NaCl concentration (in the following "0.3 M particles") is complexed with informofers the protein composition of which is simpler than that of mRNA complexes (in the following "0.1 M particles") isolated in the presence of 0.1 M NaCl. In addition, the susceptibility of "0.3 M particles" to RNase attack is more pronounced, as a consequence of which their buoyant density markedly decreases, as compared with the "0.1 M particles", if no RNase inhibitor is used during extraction (Molnár, Juhász, 1972).

The present paper described the effect of actinomycin-D on the ribonucleoprotein fractions containing dRNA extracted at 0.1 M and 0.3 M NaCl concentrations during the course of a chase following ^{14}C-orotate administration and the

effect of actinomycin-D on the incorporation of ^{14}C-orotate into the dRNA of the different nuclear ribonucleoprotein particles.

Materials and methods

In order to standardize the experiments 3 male CFY rats weighing 170 g were used in each case. The animals were given 15 μCi/100 g body weight ^{14}C-orotate (7.5 mCi/mM) intraperitoneally 35 minutes before killing or before starting the actinomycin-D chase.

Actinomycin-D (Merck) was administered intraperitoneally as described by Blobel and Potter (1967) in the case of chase in an amount of 7.5 mg/kg body weight (Georgiev et al., 1972). In the other experiments smaller doses of the drug were given.

The animals were killed in ether narcosis. The livers were homogenized in a Potter-Elvehjem type teflon-glass homogenizer in a medium containing 0.5% sodium beta-glycerophosphate and 2.2 M sucrose, and the nuclei were isolated by the modified Chauveau method (Samarina et al., 1967). The nuclear fraction was washed with STM I buffer (0.1 M NaCl – 0.01 M Tris. HCl – 0.001 M $MgCl_2$, pH 7.2), then extracted twice with STM II buffer (0.1 M NaCl – 0.01 M Tris. HCl – 0.001 M $MgCl_2$, pH 7.8), and finally twice with STM III buffer (0.3 M NaCl – 0.01 M Tris. HCl – 0.001 M $MgCl_2$, pH 7.8–8.0) (Molnár, Juhász, 1972). The acid-insoluble radioactivity of all the three fractions and of the nuclear residue was measured.

The extracts were centrifuged in a 15–30% (w/w) sucrose gradient made up in STM buffer (pH 7.5) in the 3 × 5 ml SW rotor of a Janetzki VAC-601 ultracentrifuge for 4 hours at 38 000 r.p.m. at 4°. Fractions of three drops were collected, their absorbance at 260 nm was measured, then after the addition of ribosomal RNA the material was precipitated with trichloroacetic acid, the precipitate was washed onto a nitrocellulose filter (Synpor 2), dried with ethanol, and finally radioactivity was measured in a GAMMA liquid scintillation counter, in a toluene containing apolar scintillator (100 mg PPO and 4.0 g POPOP in 1000 ml toluene).

For the electron microscopic examinations samples were taken from the liver of animals used in the experiments both in the control series and after treatment with actinomycin-D. The specimens were fixed in 2.5% glutaraldehyde diluted in 0.07 M phosphate buffer (pH 7.4) at 4° for 3 hours. Post-fixation was carried out with 2% osmium tetroxide buffered to pH 7.4 with phosphate buffer. The preparations after dehydration were embedded into araldite (CIBA) (Kay, 1965). The ultrathin sections were stained with uranyl acetate and lead citrate (Reynolds, 1963) and then examined in a TESLA BS 513 or TESLA 613 electron microscope.

Results and discussion

Two types of experiments were performed. In one series 35 minutes after the administration of ^{14}C-orotate chase was effected with a large dose of actinomycin-D and the acid-insoluble radioactivity of the individual nuclear fractions

was followed in time and the amount and labelling of nuclear ribonucleoprotein particles containing dRNA in the extracts were determined. In other experiments the incorporation of ^{14}C-orotate was registered into the dRNA-containing particles after various, but always lethal, doses of the drug. Here, too, the labelling pattern of nuclear fractions was determined. To make the results more comprehensible they are summarized in a common table and the sedimentograms on a single sheet are presented, the upper line and lower line indicating the data of "0.1 M particles" and "0.3 M particles", respectively.

We have earlier described, that the "0.1 M particles" contain 40–50% of the radioactivity incorporated into RNA, whereas the "0.3 M particles" an additional 20–30% (Molnár, Juhász, 1972). After a 160-minute actinomycin-D chase this ratio markedly changed, in particular the ratio became inverted (Table 1, experiment 2). After a 220-minute chase this tendency increased and the ratio was further shifted in favour of the radioactivity of "0.3 M particles" (Table 1, experiment 3).

Table 1

Distribution of acid-insoluble radioactivity originating from ^{14}C-orotate over the fractions of nuclear extracts and nuclear residue

Exp. 1: 35 min after the administration of 15 μCi/100 g body weight of ^{14}C-orotate the animals were killed, the nuclear fraction was prepared. Extracts were made as described in Methods and the acid-insoluble radioactivities were determined. Exp. 2: 35 min after the administration of 15 μCi/100 g body weight of ^{14}C-orotate 7.5 mg/kg body weight actinomycin-D was given. A 160-min chase. Exp. 3: 35 minutes after the administration of 15 μCi/100 g body weight of ^{14}C-orotate 7.5 mg/kg body weight actinomycin-D was given. A 220-min chase. Exp. 4: 14 hours after the administration of 2.2 mg/kg body weight actinomycin-D 15 μCi/100 g body weight of ^{14}C-orotate was given (35 min before killing the animals). Exp. 5: 17 hours after the administration of 3.2 mg/kg body weight of actinomycin-D 10 μCi/100 g body weight of ^{14}C-orotate was given (30 min before killing the animals). Exp. 6: 50 min after the administration of 4.7 mg/kg body weight of actinomycin-D 15 μCi/100 g body weight of ^{14}C-orotate was given (35 min before killing the animals).

No. of experiment	Conditions	Washing with STM III "0.1 M extract"			"0.3 M extract"		Nuclear residue	
		counts/min	counts/min	%*	counts/min	%*	counts/min	%*
1.	Control	16 200	385 570	46.6	265 940	32.1	144 480	19.3
2.	Chase with	5 427	92 288	27.3	187 740	55.5	52 340	15.5
3.	actinomycin-D	4 152	81 540	18.5	216 520	49.0	140 560	31.6
4.	Actinomycin-D +	1 428	109 692	43.8	120 000	48.3	17 720	7.2
5.	+ ^{14}C-orotate	1 920	37 920	30.8	66 000	58.7	6 880	6.8
6.		1 140	33 000	42.6	36 000	46.4	7 380	9.5

* Acid-insoluble radioactivity found in the fraction related to the total amount of incorporated acid-insoluble radioactivity

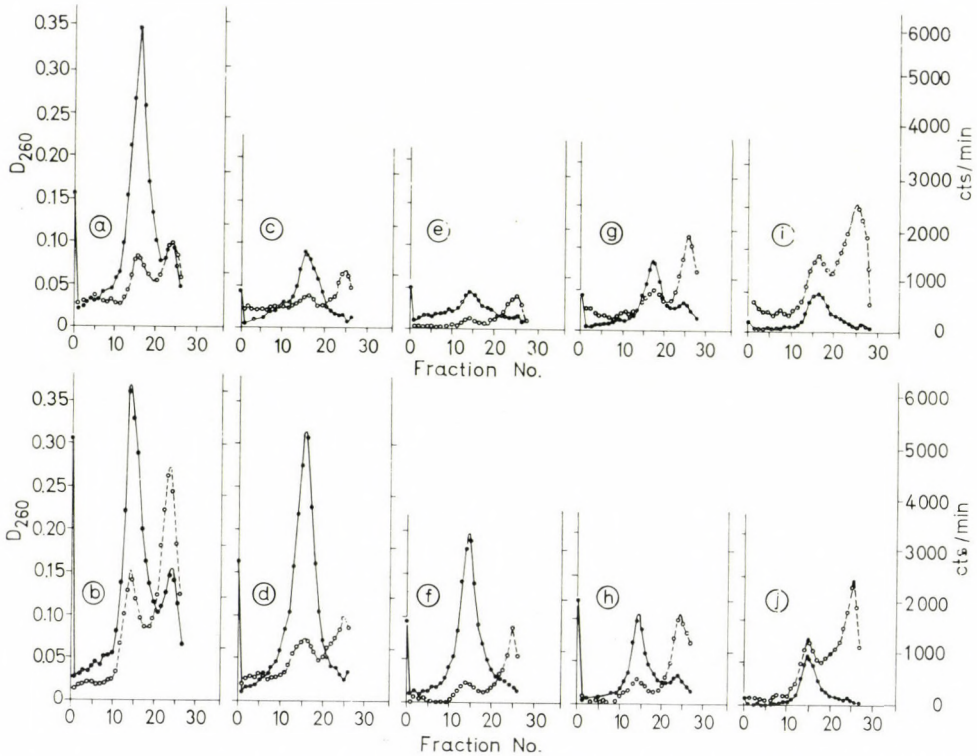

Fig. 1. Sucrose gradient ultracentrifugation of the nuclear ribonucleoprotein particles containing dRNA. The following specimens were layered on top of a 4.8 ml 15 to 30% (w/w) sucrose gradient made up in STM buffer (pH 7.5): a) 16% of control "0.1 M extract", b) 20% of control "0.3 M extract" c) 20% of the "0.1 M extract" from experiment 2 of Table 1 (160-min chase with actinomycin-D), d) 25% of the "0.3 M extract" of the same experiment, e) 20% of the "0.1 M extract" from exp. 3 of Table 1 (220-min chase with actinomycin-D), f) 20% of the "0.3 M extract" of the same experiment, g) 16% of the "0.1 M extract" of exp. 4 of Table 1 (14 hours after the administration of 2.2 mg/kg actinomycin-D 15 μCi/100 g ^{14}C-orotate was given), h) 17% of the "0.3 M extract" of the same experiment, i) 30% of the "0.1 M extract" of exp. 6 of Table 1 (50 min after the administration of 4.7 mg/kg actinomycin-D 15 μCi/100 g ^{14}C-orotate was given), j) 30% of the "0.3 M extract" of the same experiment. Sedimentation was carried out in a Janetzki VAC 601 ultracentrifuge, in the 3×5 ml SW rotor, for 4 hours at 38 000 r.p.m. and 4°. Fractions of 3 drops were collected and their absorbance at 260 nm was measured. Then rRNA was added as carrier and the material was precipitated with trichloroacetic acid, the acid-insoluble radioactivity was washed into a nitrocellulose filter and radioactivity was measured in a GAMMA liquid scintillation counter in toluene medium. o--o--o = A_{260}, • — • — • = ^{14}C radioactivity

Although it has been reported that if actinomycin-D is applied there is no 30 S peak (Samarina et al., 1966), we found an appreciable amount of radioactivity incorporated from ^{14}C-orotate (Table 1, experiment 6) even after the administration of 4.7 mg/kg body weight of the drug and the major part of radio-

activity was attached to the 30 S particles (Fig. 1, i and j). The distribution of acid-insoluble radioactivity originating from ^{14}C-orotate in the nuclear fractions was similar to that observed when the chase was applied, i.e. always the "0.3 M nuclear extract" contained more ^{14}C radioactivity (Table 1, experiments 4, 5 and 6).

At the same time significant changes could be detected in the amounts of the particles during actinomycin-D chase (Fig.1, a to f). Whereas in the case of "0.1 M particles" this decrease is very rapid and pronounced (Fig. 1, a, c and e), with the "0.3 M particles" their absolute amount fell inconsiderably smaller extent and their calculated specific activity was always higher than that of "0.1 M particles" during the chase.

The submicroscopic changes revealed after actinomycin-D administration are in accordance with the above observations; the number of interchromatic granules, the bodies homologous with "0.1 M particles" (Tigyi et al., 1970), markedly decreased on the administration of the drug (Figs 2a and 2b). The details of these experiments will be described in another paper (Komáromy et al., in preparation).

It is known about actinomycin-D that it does not inhibit the initiation of RNA synthesis, only the elongation of the chain (Goldberg, 1971). The sequences degrading inside the nucleus can be found at the 5′-end of the giant dRNA molecule (Coutelle et al., 1971) and at 0.3 M NaCl concentration these sequences are extracted (Drews, 1969). If this is so, then the sequences initiated in the presence of the drug but blocked in their elongation should be found in the "0.3 M particles".

The distribution of acid-insoluble radioactivity incorporated from ^{14}C-orotate at various actinomycin-D concentrations (Table 1, experiments 4, 5 and 6) seems to support the earlier observations according to which the "0.3 M particles" carry the dRNA sequences degraded in the nucleus. The specific activity pattern of the particles also corroborates this assumption (Fig. 1, g to j). The circumstance that the drug does not interfere with the initiation process of RNA synthesis may explain the fact that during the chase, when already 75–80% of the particle extractable with 0.1 M NaCl are already transferred to the cytoplasm (Table 1, experiments 2 and 3, and Fig. 1, a, c and e), the particles extracted with 0.3 M NaCl still contain 60 to 80% of the labelled RNA (Table 1, experiments 2 and 3 and Fig. 1, b, d and f).

It appears that during the chase the shorter and longer dRNA sequences synthesized from the ^{14}C-orotate pool may complex with informofers thus increasing the radioactivity of "0.3 M particles". This manifests itself in the enhancement of the specific radioactivity of the particles and also in the increased radioactivity of "0.3 M extract".

It should be noted that in the fraction of "0.1 M particles" along with the mRNA sequences the dRNA sequences degrading in the nucleus can also be found (Samarina et al., 1968). Since our extraction procedure does not allow the perfect separation of the two types of particles, the observations registered in the present experiments reflect only partially the actual events and thus can only indicate the main trend.

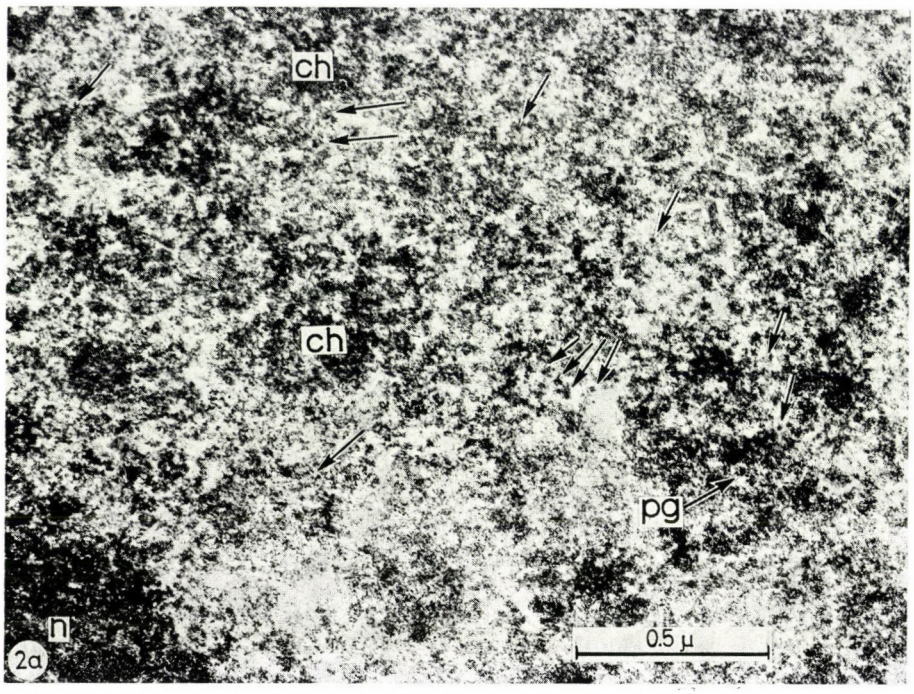

Fig. 2a. Electronmicrograph of a part of the nucleus of a rat liver cell. Control. A great number of interchromatic granules (indicated by arrows) can be discerned in the interchromatic substance of the nucleus. n : part of the nucleolus, ch : diffuse chromatin, pg : perichromatic granule

At any rate, the experiments described in this paper provide further support to our earlier notion, i.e. that the informofer is heterogeneous (Molnár, Juhász, 1972). One of its fractions, the "0.1 M informofer", forms complexes with mRNA, the other, the "0.3 M informofer" complexes with the dRNA sequences that are degraded inside the nucleus.

The fractionated extraction renders it possible to prepare a ribonucleoprotein fraction rich in mRNA molecules and another fraction rich in dRNA sequences degraded in the nucleus.

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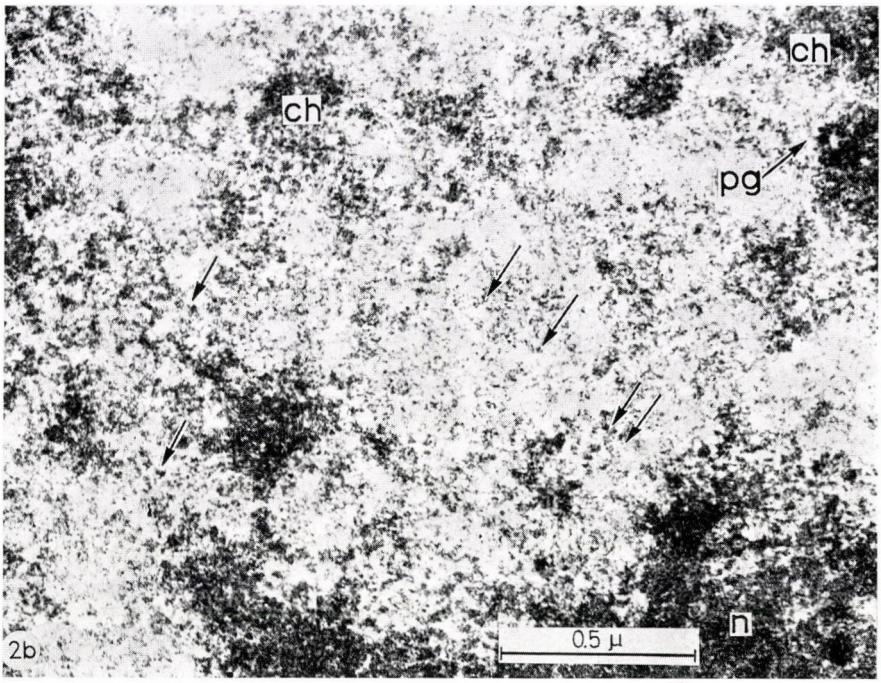

Fig. 2b. Electron micrograph of a part of the liver cell nucleus of rat treated with 2.2 mg/kg of actinomycin-D. The amount of interchromatic granules (arrows) in the interchromatic substance decreased as compared with the control. n : nucleolus, ch : diffuse chromatin, pg : perichromatic granule. Magnification: 50 000×.

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Tracer Kinetic Analysis of Phosphate Incorporation of Erythrocytes in vitro

II. Model Analysis of the System with the ATP Pool not in Steady State

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It has been observed in experiments *in vitro*, that the acid-labile phosphate pool of intact erythrocytes may not be in the steady state even under normal metabolic conditions. The determination of ATP in erythrocytes in such experiments by the firefly luciferin-luciferase method has shown that the cause of the non-steady state behavior of the acid-labile phosphate pool is the decrease of ATP in the cell.

Tracer kinetic model analysis of these cases has established that experiments with the non-steady state acid-labile phosphate pool can be described by the model outlined in our previous paper. However, at the very end of these experiments, where specific activity curves of the extracellular P_i and acid-labile phosphate intersect, the model fails to describe the system. The heterogeneity of the acid-labile pool may be the reason for this failure of the model.

Introduction

In our previous work (Latzkovits et al., 1972) we found a four-compartment model, which satisfactorily described the process of phosphate incorporation into erythrocytes *in vitro*. The four compartments are as follows

A : Extracellular P_i

B : Intracellular P_i

C : Intracellular organic acid-labile phosphate

D : Intracellular organic acid-resistant phosphate.

The validity of the model, however, was checked only for those experiments in which compartments C and D were in the steady state. But in about 10% of the experiments we observed a decrease of the acid-labile pool in intact erythrocytes from healthy donors under adequate metabolic conditions. In these cases, the tracer kinetic model analysis seemed to be very important from the point of view of verifying the compartmental model. By this way one can check the validity of the model by testing its applicability under modified conditions of the system. Therefore we employe the tracer kinetic model analysis for the exheriments in which the acid-labile phosphate decreased. Further, we investigated whether the decrease of the acid-labile phosphate pool is only due to the decomposition of ATP, as might be expected, or whether the kinetics of the decrease of other acid-labile compounds should also be determined. The mechanism of the decrease was not studied since it bears no significance to the present model analysis.

Materials and methods

Materials and methods used in these experiments are the same as described earlier (Latzkovits et al., 1972). In addition, ATP was determined by the firefly luciferin-luciferase method according to Strehler and Totter (1952). Samples were taken from the suspension at different times of incubation. The erythrocytes were washed and precipitated with 6% perchloric acid (PCA), and the PCA supernatant was neutralized by precipitating PCA with KOH. The measurement of light emission was performed in a Packard Tri-Carb spectrometer (type 3375). The crude desiccated luciferin-luciferase of firefly tails was provided by Sigma Chemical Corp.

Mathematical treatment

The mathematical principles and procedures are the same as outlined in the previous paper (Latzkovits et al., 1972). However, in this paper the process of the decrease of the acid-labile pool will be taken into account in the kinetic description. Considering that this process results in a further increase of intracellular P_i, we can kinetically describe it as a "transport" process from the acid-labile phosphate compartment to the intracellular P_i compartment (Solomon, 1960). This "transport" process is reflected in the change of the amount of acid-labile phosphate. Thus each of the logically possible compartmental models representing the system with the non-steady state acid-labile pool, should include two transport processes beside the exchanges: one is the transport of P_i from the extracellular P_i pool to the interior of the cell across the membrane, and the other is an "abstract transport" representing the decomposition of the acid-labile pool. Consequently, the Branson equations (1961) can be set up as follows:

$$\frac{dI_A}{dt} - \frac{dA}{dt} S_A = W_{AB}(S_B - S_A) + W_{AC}(S_C - S_A) + W_{AD}(S_D - S_A) \quad (1)$$

$$\frac{dI_B}{dt} + \frac{dA}{dt} S_A + \frac{dC}{dt} S_C = W_{AB}(S_A - S_B) + W_{BC}(S_C - S_B) + W_{BD}(S_D - S_B) \quad (2)$$

$$\frac{dI_C}{dt} - \frac{dC}{dt} S_C = W_{AC}(S_A - S_C) + W_{BC}(S_B - S_C) + W_{CD}(S_D - S_C) \quad (3)$$

$$\frac{dI_D}{dt} = W_{AD}(S_A - S_D) + W_{BD}(S_B - S_D) + W_{CD}(S_C - S_D) \quad (4)$$

where I_A, I_B, I_C and I_D are total activities of pools A, B, C and D (cpm per ml washed red blood cell (RBC) or per 4 ml Krebs–Ringer solution); S_A, S_B, S_C and S_D are specific activities of the four pools, A – D (cpm per μg phosphorus); W_{XY} is the rate of exchange between the two pools marked by indices (μg phosphorus per min per ml washed RBC or 4 ml Krebs–Ringer solution; by definition the values of W_{XY} must be positive or zero); and A, B, C and D the amount

of phosphate of particular pools (μg phosphorus per ml washed RBC or 4 ml Krebs–Ringer solution).

It can be seen that the "transport" process representing the decrease of the acid-labile phosphate pool is given by $(dC/dt)S_C$ in equations (2) and (3).

Results

The results concerning the quantitative distribution over the four pools are demonstrated in Fig. 1 and Table 1. It can be seen from Table 1 that pool

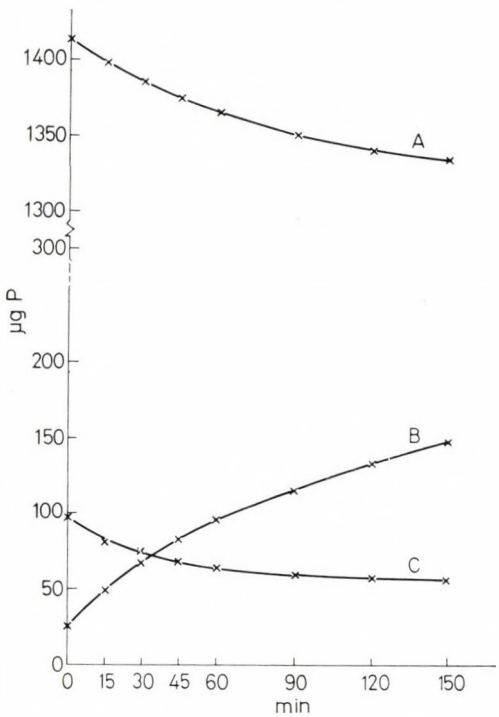

Fig. 1. Changes in the amount of the extra- and intracellular P_i and the acid-labile phosphate in 4 ml of Krebs–Ringer solution and 1 ml of washed RBC, respectively, versus time. A: extracellular P_i; B: intracellular P_i; C: acid-labile phosphate. Each of the points represents the mean value of 10 experiments. Standard deviations are within 6%

D is in a steady state. The material balance of the changes in the amount of the A, B and C pools in Fig. 1 can be stated as follows:

$$\frac{dA}{dt} + \frac{dB}{dt} + \frac{dC}{dt} = 0. \tag{5}$$

Table 1

Amount of acid-resistant phosphate (pool D) during incubation

Time of incubation min.	μg P/ml washed RBC ± standard deviations
0	298±15*
15	305±19
30	285±20
45	290±11
60	301±14
90	307± 9
120	300±16
150	296±12

* Mean values and standard deviations are calculated from the data of 10 experiments.

This material balance, demonstrated in Table 2, is in good agreement with the above-mentioned assumption, i.e. that the changes in the amounts of pools A, B and C can be given in the kinetic description as the results of two "transport" processes: one is the transport of P_i across the membrane of the cell from the medium and the other is an "abstract transport" representing the decomposition of pool C.

The change in the amount of ATP was simultaneously determined by the firefly luciferin-luciferase method. The results in Fig. 2 show that the decrease

Table 2

Material balance of extracellular and intracellular inorganic and acid-labile phosphate pools

Time of incubation, min.	$\left(\frac{\frac{dA}{dt}+\frac{dC}{dt}}{-\frac{dB}{dt}}-1\right)\times 100$
0	4.04
15	−2.25
30	3.36
45	−2.88
60	−3.69
90	−1.74
120	−2.59
150	3.47

of ATP parallels the decrease of pool C. Table 3 gives the amount of the non-ATP subfraction of pool C. It can be seen that this subfraction is in the steady state, and at zero time it amounts to about 20% of pool C. Thus we can establish that the decrease of pool C is only due to the decomposition of ATP.

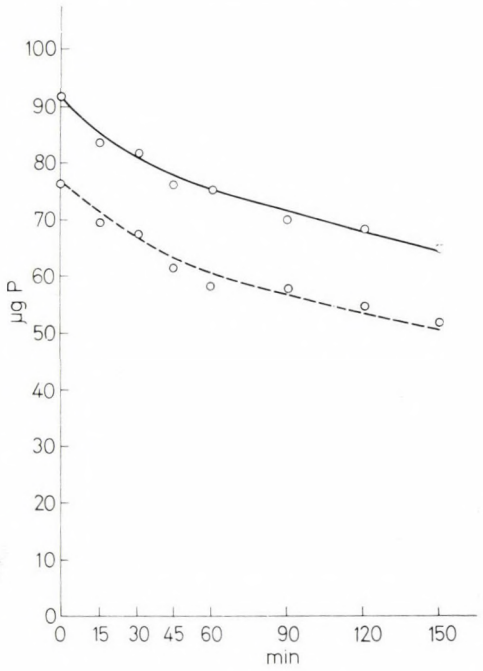

Fig. 2. Changes in the amount of the acid-labile phosphate and of pyrophosphate in ATP as determined by the firefly luciferin-luciferase method in 1 ml of washed RBC versus time. Solid line: the acid-labile phosphate; dotted line: the pyrophosphate in the ATP. Each of the points represents the mean value of 10 experiments. Standard deviations are within 6%

The kinetics of the two "transport" processes were investigated by a curve-fitting procedure with a digital computer (type Minsk 22). The same equation described previously (Latzkovits et al., 1972) was obtained for the P_i transport across the membrane. The "transport" from compartment C to compartment B can be described by the following equation:

$$-\frac{dC}{dt} = 0.3\, e^{-0.008t}. \tag{6}$$

Our results concerning the changes in the total and specific activities of the four pools are demonstrated in Figs 3 and 4. Comparing the total activity curves of all four pools, as well as the specific activity curves of pools A, B and D, to those obtained in the first part, we find no significant differences in the nature of

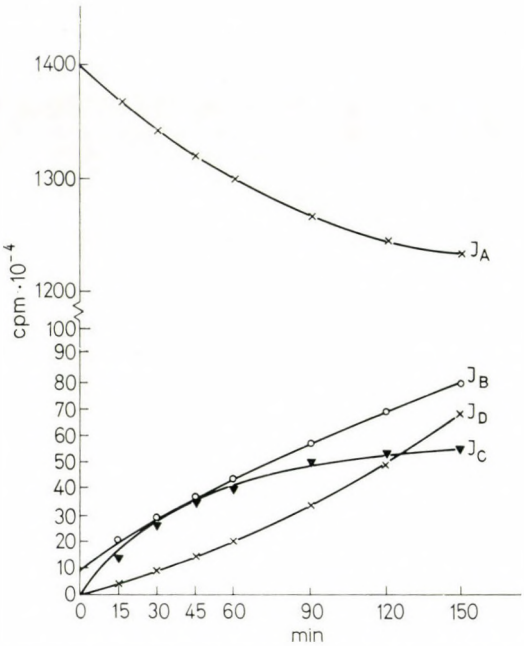

Fig. 3. Total activity curves of the phosphate pools in 1 ml of washed RBC and in 4 ml of the Krebs–Ringer solution, respectively, versus time. I_A: extracellular P_i pool; I_B: intracellular P_i pool; I_C: acid-labile phosphate pool; I_D: acid-resistant phosphate pool. Each of the points represents the mean value of 10 experiments. Standard deviations are within 5%

Table 3

Amount of phosphate in the non-ATP subcompartment of the acid-labile phosphate pool

Time of incubation, min.	μg P/ml washed RBC ± standard deviation
0	15±2.3*
15	11±1.8
30	19±1.0
45	14±1.5
60	12±2.1
90	16±1.1
120	14±2.0
150	17±1.7

* Mean values and standard deviations are calculated from the differences between the amount of acid-labile phosphate and of ATP pyrophosphate as determined by the firefly luciferin-luciferase method in 10 experiments.

the curves. However, it is rather conspicuous that the specific activity curve of pool C is steeper even than the curve of the previous experiments with pool C in the steady state, and the S_A and S_C curves intersect at the end of the experiments.

The data included in Figs 3 and 4 enable us to apply model analysis in the same way as in the previous paper, because the validity of the applied relations does

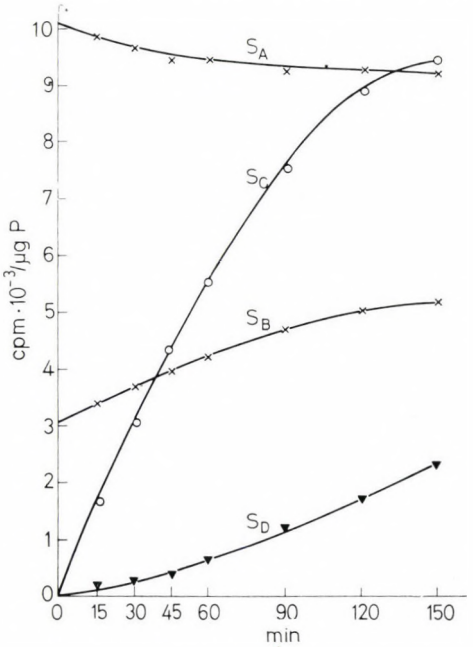

Fig. 4. Specific activity curves of the phosphate pools. S_A: extracellular P_i; S_B: intracellular P_i; S_C: acid-labile phosphate; S_D: acid-resistant phosphate. Each of the points represents the mean value of 10 experiments. Standard deviations are within 5%

Table 4

Exchange rate values of the isomorphous model
(μg P/min/ml washed RBC)
Rate values were calculated from the data of Figs 1, 3 and 4.

Time of incubation, min.	W_{AC}	W_{BC}	W_{BD}
0	0.926	1.410	0.569
15	1.071	3.047	0.850
30	1.012	2.738	1.032
45	1.223	1.003	1.164
60	1.380	1.562	1.301
90	3.117	1.874	1.322
120	7.341	1.792	1.371
150	−5.653	1.060	1.586

not change with the present numerical data. As a result of the model analysis we could establish the same model to be isomorphous with that obtained previously. The numerical rate values of the exchanges for the present experiments are given in Table 4. It is apparent from Table 4, that the rate values of W_{AC} are not constant after 90 min; even toward the end of the experiments where the S_A and S_C curves intersect, W_{AC} becomes negative.

Discussion

The fact that irrespective of the instability of pool C in erythrocytes the tracer kinetic model analysis of the distribution of labelled P_i in the system studied provides the same compartmental model as reported for the experiments with pool C being in the steady state, supports our preliminary assumption that the *in vitro* system of P_i transport and metabolism in erythrocytes can indeed be described in terms of the four compartments. However, one must question why, at the very end of the present experiments, W_{AC} becomes negative, so that the model no longer describes the system. Comparing the present results with those obtained previously we can state that this failure of our model can be ascribed to the decrease of pool C. Since ATP is the only compound that is decomposed here (Fig. 2 and Table 3), this pool consists of both a constant and a decreasing subcompartment. That is, the fact that ATP is not in the steady state in the present experiments, renders the heterogeneity of pool C manifest.

It is to be mentioned that the role of the heterogeneity of the ATP pool itself cannot be excluded (Szász, 1970; Welt et al., 1963).

The aim of our present work was to study simultaneously the phosphate transport and exchange across the membrane, in connection with metabolism. The disturbing effect of the heterogeneity of pool C becomes manifest at the end of these experiments. That is the reason why, at present, we do not take this heterogeneity into account according to the principle of "economy" of model constructions as formulated by A. K. Solomon, which prescribes the construction of compartmental models by including the possible smallest number of compartments (Solomon, 1960).

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Localization of a so far Unidentified Cysteinyl Residue in Rabbit Muscle Aldolase

(Short Communication)

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Seven peptides containing carboxymethyl cysteine have previously been isolated by us from the tryptic hydrolysate of fully carboxymethylated rabbit muscle aldolase (Sajgó, 1969). Similarly, seven peptides containing carboxymethyl cysteine have been found by Anderson and Perham (1970), which proved to be identical with those described by us.

However, in the radioautograph of the tryptic hydrolysate of the N-terminal cyanogen bromide fragment (CB-1) (cf. ref. Sajgó, 1971) of fully carboxymethylated aldolase in addition to the two very distinct tryptic peptides containing carboxymethyl cysteine described earlier (N2: Cmc.Val.Leu.Lys, and N3: Cmc. Ala. Gln. Tyr. Lys), there appeared a few radioactive components, not in every case, somewhat blurred and adsorbed to the start line which contained carboxymethyl cysteine, were free of basic amino acids, and could not be fitted into any of the known peptide sequences. On this basis it was reasonable to assume that there was another structural portion containing a further (the 8th) cysteinyl residue, which was located in the N-terminal cyanogen bromide fragment. Anderson and Perham (1970), as well as Anderson (1972) came to the same conclusion. They isolated a cysteine-containing peptide not yet characterized, from the combined tryptic and chymotryptic hydrolysate of the N-terminal fragment obtained by cyanogen bromide cleavage, and determined the N-terminal amino acid and the amino acid composition. Lai et al. (1971) also refer to the isolation of a similar structural segment without providing data about the amino acid composition.

In the course of the study of the primary structure of aldolase we succeeded in identifying a peptide comprising 20 amino acid residues, among them the 8th cysteine, during the analysis of radioactive minor components that occur in the tryptic hydrolysate of CB-1 fragment and also during the analysis of one of the radioactive peptides isolated from the chymotryptic hydrolysate of CB-1 fragment.

Aldolase fully carboxymethylated with [^{14}C]-bromoacetate was cleaved by cyanogen bromide. As described earlier (Sajgó, 1971), the acylated N-terminal cyanogen bromide fragment (CB-1) was isolated and deacylated. Tryptic hydrolysis was allowed to proceed for 1.5 hours with 1/50 (weight/weight) trypsin in 0.1 M ammonium hydrocarbonate, pH 8.5, at 37°C. Hydrolysis was stopped by the addition of di-isopropyl fluorophosphate, the hydrolysate was freeze-dried and

analysed by means of two-dimensional electrophoresis (in pyridine–acetic acid, pH 6.5, and acetic acid–formic acid, pH 1.9, buffers) followed by radioautography. The radioautograph revealed, in addition to the spots of N2 and N3, two faint acidic components (A-I and A-II) and another component (NS) which remained at the start (Fig. 1). The latter component could not yet be prepared in appropriate purity, whereas components A-I and A-II were purified by preparative electrophoresis, and their amino acid composition and sequence were determined. The amino acid composition was determined by the single-column procedure of Dévényi (1969), the sequencing of N-terminal portion was performed by the dansyl-Edman method (Gray, 1967). The C-terminal sequence was determined

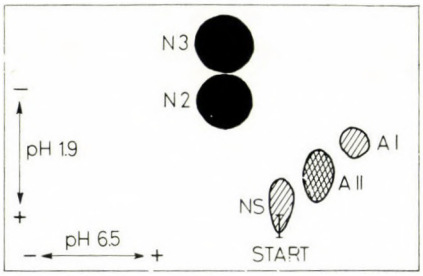

Fig. 1. Tracing of the radioautograph of the tryptic hydrolysate of the CB—1 fragment of aldolase carboxymethylated with [^{14}C]-bromoacetate after two-dimensional electrophoresis (1: pyridine–acetic acid–water, 96 : 4 : 900, v/v, pH 6.5; 2: formic acid– acetic acid–water, 20 : 80 : 900, v/v, pH 1.9; voltage gradient 50 and 75 volts/cm, respectively)

by digestion with carboxypeptidase A + B, the split amino acids were identified by the aid of thin-layer ion exchange chromatography (Dévényi et al., 1971; Sajgó, Dévényi, 1972). Fig. 2 shows the amino acid composition of samples taken at different times during digestion with carboxypeptidase of peptide A-II, as assayed by thin-layer ion exchange chromatography.

The sequence analysis of peptides A-I and A-II gave the following results:

A-I: Val. (Asn_1*Pro_1 Cmc_1)

A-II: Val.Asn*.Pro.Cmc.Gly.Gly.Ile.Val.Ile.Leu.Phe

In the chymotryptic hydrolysate of fragment CB-1 (1/50 w/w chymotrypsin, in 0.1 M ammonium hydrocarbonate, pH 8.5, 37°C, 2 hours) several radioactive components could be found. The amino acid analysis of one of the components, which had anodic mobility at pH 6.5, suggested that this peptide comprises peptide A-II with the exception of Phe. The peptide was purified by electrophoresis and chromatography and its sequence was determined:

Thr.Ala.Asp.*Asp.*Arg.Val.Asn*Pro.Cmc.Gly.Gly.Ile.Val.Ile.Leu

The segment Thr to Arg of this peptide is identical with the C-terminal sequence

* Determined on the basis of electrophoretic mobility.

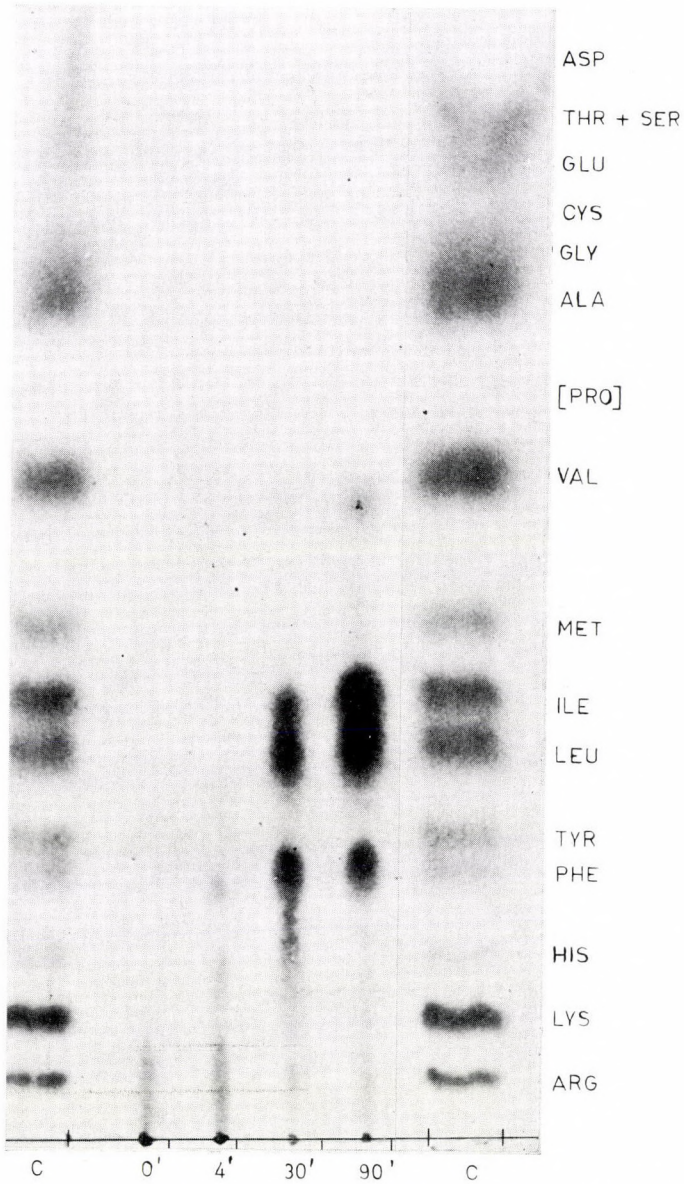

Fig. 2. Thin-layer ion exchange chromatograms of samples taken at 0, 4, 30 and 90 min during digestion with carboxypeptidase A + B of peptide A—II at 37°C. (Ionex 25-SA Na^+ chromatoplate, Macherey-Nagel and Co., Düren; at 50°C, in 0.4 M Na^+, 0.4 M citrate buffer, pH 3.3). C = control

of a peptide (Gln.*Leu.Leu.Leu.Thr.Ala.Asp*Asp*Arg) isolated earlier from the tryptic hydrolysate of fragment CB-1. On the basis of the overlap we assume that peptide A-II, which contains the 8th cysteinyl residue, is aligned to this peptide

in the polypeptide chain of the protein, thus the sequence of this portion reads:

Gln.Leu.Leu.Leu.Thr.Ala.Asp.Asp.Arg.Val.Asn.Pro.Cmc.Gly.Gly.Ile.Val.Ile.Leu. Phe.

The presence of peptides A-I and A-II in the tryptic hydrolysate indicates that these peptides are formed partly by the splitting, according to the specificity of trypsin, of the ... Arg.Val ... bond, and partly, as a result of the inherent chymotryptic-like activity of trypsin, by the splitting of the Phe.X ... and Cmc. Gly ... bonds, which are hypersensitive to chymotryptic attack.

If pure chymotrypsin or a mixture of trypsin and chymotrypsin is used, already the ... Leu.Phe ... bond is split and in this case Leu will be the C-terminus.

As it is apparent from the sequence presented, this segment of the polypeptide chain contains an unusually great number of nonpolar amino acid residues clustered together. The problems encountered in the isolation and purification of these particular peptides can presumably be attributed to this circumstance.

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Symmetry and Asymmetry in the Mechanisms of Hydrolysis by Serine Proteases and their Thiol Analogues

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According to the symmetry postulate in the reaction of serine proteases with alkyl oxygen esters, acylation and deacylation of the intermediate acyl-enzyme proceed by the same mechanism (Bender, Kézdy, 1965). The symmetry of acylation and deacylation is tested here in the following reactions:

1. serine enzyme with alkyl oxygen ester
2. serine enzyme with alkyl thiol ester
3. thiolenzyme with alkyl oxygen ester
4. thiolenzyme with alkyl thiol ester.

Subtilisin and chymotrypsin were used as serine enzymes. The thiolenzyme was a thiol derivative of subtilisin in which the serine residue at the active site was replaced by a cysteine residue. Nonspecific and specific substrates, like the alkyl ester, the alkyl thiol ester and the p-nitrophenyl ester derivatives of acetic acid, cinnamic acid, Z-glycine and N-acetyl phenylalanine, were employed.

In the reaction of serine proteases with alkyl oxygen esters, symmetry of acylation and deacylation holds only if general base and general acid catalyses but not if the stereochemistry of the reactions is considered. In the case of thiol esters, the lack of symmetry of acylation and deacylation is indicated by the fact that the rate of *acylation* of Carlsberg subtilisin by alkyl thiol ester substrates is significantly higher than the rate of acylation by the corresponding oxygen esters, whereas the rate constants for *deacylation* of acetyl- and cinnamoyl-thiolsubtilisin are much lower than those of the same derivatives of the serine enzyme.

The rate constants of enzymatic and alkaline hydrolysis of esters and thiol esters were also compared. In contrast to the enzymatic hydrolysis, the alkaline hydrolyses of esters and thiol esters proceed at a similar rate. This indicates that the tetrahedral intermediate formed in the enzymatic hydrolysis is different from the simple additional intermediate formed in the alkaline hydrolysis.

The contribution of orientation of the reacting groups to the catalysis is discussed. It is stated that orientation and rigidity cannot be treated separately.

The detailed catalytic mechanism of serine proteases may depend not only on the chemical nature of the leaving group but also on the specificity, i.e., on the mode of binding, of the substrate.

Introduction

Our present knowledge about the mechanism of action of serine proteases is mainly based on studies of the reaction of chymotrypsin with oxygen ester substrates. These studies established that the formation and the hydrolysis of the intermediate acyl-enzyme are mechanistically symmetrical processes (cf. Bender, Kézdy, 1965). By using substrates of different type, one may expect that some additional features of the catalysis could be revealed. In this paper the kinetics

of the reaction of alkyl thiol esters is investigated. The study of the hydrolysis of alkyl thiol esters is neglected in the literature. It has been shown only qualitatively that chymotrypsin is able to hydrolyze ethyl N-acetyl-DL-thiol-phenylalaninate (Goldenberg et al., 1950).

In the first place we shall present kinetic data of the reaction of the serine protease, subtilisin type Carlsberg, with alkyl thiol esters, like ethyl thiolacetate, ethyl thiolcinnamate, and ethyl Z-thiolglycinate. Subtilisin was preferred to chymotrypsin for the following reason. Subtilisin can be transformed into thiolsubtilisin by replacing the serine side chain at the active site for a cysteine residue (Polgár, Bender, 1970). The thiolenzyme obtained in this way hydrolyzes alcohol-activated ester substrates, like p-nitrophenyl acetate. The hydrolysis takes place through the formation of an acyl-thiolenzyme intermediate. This renders it possible to study the symmetry of acylation and deacylation processes when the thiol ester bond is hydrolyzed. In other words, the effect of the replacement of oxygen by a sulfur atom can be studied both in acylation (acylation of serine enzyme with thiol esters) and in deacylation (hydrolysis of acyl-thiolenzyme).

The following reaction types were studied: 1. serine enzyme with alkyl oxygen ester, 2. serine enzyme with alkyl thiol ester, 3. thiolenzyme with alkyl oxygen ester, 4. thiolenzyme with alkyl thiol ester. The comparison of these reactions allowed to conclude that the mechanism of acylation and deacylation is symmetrical only in case 1, if general base and general acid catalyses are considered as criteria for symmetry. However, the stereochemistry of acylation and deacylation is not symmetrical even for the reaction of serine proteases with alkyl oxygen esters.

In the present work we employed Carlsberg subtilisin, since this highly active enzyme is more convenient for measuring the hydrolysis of poor substrates although some of the reactions (case 3) have already been studied with the Novo enzyme (Polgár, Bender, 1967). These earlier investigations with the Novo thiolenzyme are now extended to the Carlsberg thiolsubtilisin.

Materials and methods

Substrates

Ethyl acetate was purchased from Reanal, p-nitrophenyl Z-glycinate from Cyclo Chemical Co.

p-Nitrophenyl acetate and cinnamate were synthetized from p-nitrophenol and the corresponding acid by means of dicyclohexyl carbodiimide as described for amino acid nitrophenyl esters (Bodánszky, du Vigneaud, 1962). The reaction was performed in dimethyl-formamide as solvent. p-Nitrophenyl acetate was recrystallized from ethanol, m.p. 78 – 79°, p-nitrophenyl cinnamate from chloroform-cyclohexane, m.p. 145 146°.

Ethyl cinnamate and ethyl thiolacetate were prepared as described by Peters (1929) and Seifert (1885), respectively. Ethyl N-acetyl-DL-thiol phenylalaninate was prepared according to the method of Goldenberg et al. (1950).

Ethyl thiolcinnamate was synthetized as follows. Cinnamoyl chloride was prepared from cinnamic acid and thionyl chloride. Cinnamoyl chloride (10 g) was dissolved in pyridine (15 ml) and ethyl mercaptane (4 g) was added to the cold solution. After two days standing at room temperature, it was poured into a mixture of ice and 20% sulfuric acid (1 : 1). The product was extracted into ether, washed with $NaHCO_3$, and dried over Na_2SO_4. Ether was removed *in vacuo* and the product distilled at 125–126°, 16 Hgmm.

Ethyl Z-thiolglycinate was prepared by reacting ethyl mercaptane with the mixed anhydride of Z-glycine and formic acid. The mixed anhydride was prepared according to the method of Wieland and Bartmann (1956) and was allowed to stand for 5 days at room temperature with an excess of ethyl mercaptane. The reaction mixture was then poured into water. The thiol ester was separated as an oil, was extracted into ether and after washing with water, the solution was dried with Na_2SO_4. Ether and the excess of mercaptane were removed *in vacuo*. The residual colorless oil had an absorption spectrum characteristic of thiol ester.

Ethyl-Z-glycinate was prepared by professor K. Medzihradszky for us. N-acetyl-L-phenylalanine ethyl ester was a gift of Dr T. Vajda.

Enzymes

Subtilisin type Carlsberg and α-chymotrypsin were products of Novo Industri A/S, and Sigma Co., respectively.

Preparation of Carlsberg thiolsubtilisin for kinetic studies. The preparation of Carlsberg thiolsubtilisin was reported earlier (Polgár, 1958). This enzyme is not suitable for kinetic investigations since it cannot be completely inhibited with SH-reagents. For example, the hydrolysis of p-nitrophenyl acetate catalyzed by Carlsberg thiolsubtilisin is only inhibited about 60% with p-mercuribenzoate. However, if the enzyme is preincubated with 0.05 mole equivalents of phenylmethane-sulfonyl fluoride, a potent inhibitor of subtilisins, the inhibition by p-mercuribenzoate is practically complete. This indicates that during the preparation of the thiolenzyme, when the inactive phenylmethanesulfonyl subtilisin is incubated with thiolacetate not only the thiolenzyme is formed but also a small amount (less than 5%) of the original serine enzyme is regenerated. It is this contamination that is not inhibited by p-mercuribenzoate. Since subtilisin displays a significantly higher specific activity toward p-nitrophenyl acetate than does its thiol-derivative, traces of the serine enzyme can falsify the determination of the kinetics of thiolenzyme.

It should be noted that phenylmethanesulfonyl subtilisin type Novo is more resistant to hydrolysis than this derivative of the Carlsberg enzyme. Novo thiolsubtilisin is sufficiently pure to allow kinetic investigations without preincubation with phenylmethanesulfonyl fluoride.

Cinnamoyl-subtilisin and cinnamoyl-thiolsubtilisin type Carlsberg. These derivatives of subtilisin were prepared as described for the corresponding derivatives of the Novo enzyme (Polgár, Bender, 1967).

Determination of enzyme concentration. The concentration of the active serine enzymes was determined by titrating their active sites with N-trans-cinnamoyl-imidazole (Polgár, 1968; Schonbaum et al., 1961). The concentration of the thiolenzyme was measured by titrating with p-mercuribenzoate (Boyer, 1954).

Kinetic measurements

The kinetics of the reactions were measured at 25.0° in Spektromom 202 or Opton DMR 21 spectrophotometers fitted with thermostated cell compartments. The substrates were dissolved in acetonitrile which was purified according to the method of Lewis and Smith (1939). The reactions were run in the presence of 1.7% acetonitrile in 0.1 M phosphate or carbonate buffer.

The release of nitrophenol and nitrophenolate ion during the reaction of p-nitrophenyl esters was followed at 348 and 400 nm, respectively. The hydrolysis of ethyl cinnamate was measured at 300 nm or when high ester concentration was used, at 312 nm.

The hydrolysis of ethyl thiolcinnamate was followed at 310 nm and the release of cinnamate ion at 250 nm. The kinetics of the hydrolysis of ethyl thiolacetate, ethyl Z-thiolglycinate, and ethyl N-acetyl-DL-thiolphenylalaninate was determined at 245, 235 and 240 nm, respectively. In the case of ethyl thiolacetate, where high concentration of subtilisin had to be used, the same amount of enzyme was employed in the sample and the reference cells.

The concentration of ethyl acetate, ethyl Z-glycinate, and ethyl N-acetyl-L-phenylalaninate was measured by the alkaline hydroxamic acid test (Hestrin, 1949).

Correction was made for spontaneous hydrolysis of labile esters.

The alkaline hydrolysis of esters was monitored similarly to the assay method used in the presence of enzymes. The hydrolysis of ethyl thiolacetate and ethyl Z-thiolglycinate was an exception since the absorption at 250 nm increased rather than decreased due to the high absorption of the mercaptide ion formed in the alkaline medium. The reaction rate of these two thiol esters was determined in the presence of 0.002 M EDTA in stoppered cells completely filled with the reaction mixture.

The kinetic constants used in this paper are defined by equation 1, which shows the steps of the catalysis with serine proteases (cf. Bender, Kézdy, 1965).

$$E + S \overset{K_S}{\rightleftharpoons} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} \begin{matrix} E + P_2 \\ + P_1 \end{matrix} \qquad (1)$$

where K_S is the dissociation constant of ES, the enzyme-substrate complex, ES′ is the acyl-enzyme and P_1 and P_2 stand for the leaving group and for the carboxylic acid, respectively.

The reactions pseudo first-order with respect to the substrate were measured under conditions when $S < K_m$. The second-order rate constants were obtained by dividing the first-order rate constants by the concentration of the active en-

zyme. The second-order rate constant calculated in this way is equal to $k_2/K_S = k_{cat}/K_m$ (cf. Bender, Kézdy, 1965). In the case of chymotrypsin k_{cat}/K_m could also be calculated directly from the Lineweaver-Burk plot.

The reaction conditions for acylation of Carlsberg subtilisin were as follows: 5.0×10^{-3} M ethyl acetate and 2.3×10^{-4} M subtilisin; 4.2×10^{-4} M ethyl thiolacetate and 8.5×10^{-5} M subtilisin; 2.2×10^{-3} M ethyl Z-glycinate and $(4.9-10.3) \times 10^{-6}$ M subtilisin; 4.2×10^{-4} M ethyl Z-thiolglycinate and $(1.2-7.5) \times 10^{-7}$ M subtilisin; 1.0×10^{-4} M p-nitrophenyl Z-glycinate and 1.6×10^{-7} M subtilisin.

Results

Acylation of serine proteases with thiol and oxygen esters

Acylation of Carlsberg subtilisin by ethyl thiolcinnamate, ethyl and p-nitrophenyl cinnamate. Using cinnamoyl ester substrates one can measure not only the splitting of the ester bond but also the hydrolysis of the acyl-enzyme by measuring the formation of the cinnamate ion (Bender et al., 1962). Thus we studied the hydrolysis of ethyl thiolcinnamate at 310 and 250 nm, corresponding to the splitting of the thiol ester bond and the release of P_2 of equation (1) respectively. The reaction followed pseudo first-order kinetics at least up to 90% and the rate constants proved to be identical within experimental error at both wavelengths (Table 1). This indicates that acylation is rate-determining under the conditions of the experiments. This is in agreement with the fact that the firts-order rate constant measured even at the highest enzyme concentration is much lower ($E_0 = 2.44 \times 10^{-4}$ M, pH = 7.6, $k^{1st} = 5.25 \times 10^{-3}$ sec^{-1}) than the rate constant of deacylation of cinnamoyl-subtilisin (0.20 sec^{-1}, see Fig. 4).

The data presented in Table 1 also show that the second-order rate constants are independent of both the enzyme and the substrate concentration. This implies that the reaction rates are proportional to the substrate concentration and thus the enzyme is far from saturation, i.e. $S < K_m$, even at the highest substrate concentration (9.5×10^{-5} M).

Fig. 1 shows the pH-dependence of the acylation of Carlsberg subtilisin by ethyl thiolcinnamate. The pH-independent maximal rate constant, k(limit), and the pK_a of the ionizable group on which the reaction depends are calculated from the theoretical curve and are compiled in Table 2.

For the sake of comparison, the hydrolysis of the oxygen esters, ethyl and p-nitrophenyl cinnamate, catalyzed by subtilisin was also measured. In this way the effect of the character of the leaving group on the reaction could be tested since in the pH-range of the measurements the mercaptide ion is a better leaving group than the alkoxide ion, and the nitrophenolate ion is a still better one. It should be noted, however, that the leaving ability of a group does not necessarily manifest itself in an acylation reaction because the formation of the addition intermediate may be rate-limiting, as it will be seen below in the case of the alkaline hydrolysis of esters and thiol esters. Of course, a strong electron-withdrawing

Table 1

*The second-order rate constants of the reaction of Carlsberg subtilisin with ethyl thiolcinnamate**

pH	Enzyme $M \times 10^5$	Substrate $M \times 10^5$	Wavelength of the measurement nm	k $M^{-1} sec^{-1}$
6.51	1.13	2.16	310	4.42
			250	4.07
6.95	1.33	2.16	310	9.75
			250	11.5
7.55	1.37	2.16	310	23.3
			250	22.2
8.00	1.27	2.16	310	43.2
			250	39.4
8.50	1.29	2.16	310	43.3
			250	38.0
9.05	1.29	2.16	310	44.6
			250	49.5
7.60	2.44	2.71	310	21.5
7.60	4.88	2.71	310	21.5
7.60	12.2	2.71	310	22.4
7.60	24.4	2.71	310	21.5
7.55	2.46	2.38	310	24.4
7.55	2.74	4.75	310	22.7
7.55	2.64	9.50	310	26.0

* 25.0° 0.1 M phosphate or carbonate buffer.

group, like the p-nitrophenyl group, may also affect the rate-limiting first step by increasing the positive charge on the carbonyl carbon atom.

The pH-dependence of the rate constants of ethyl and p-nitrophenyl acetate is shown in Fig. 2 and the kinetic parameters calculated from the theoretical curves in Table 2. It is seen that the second-order rate constant of acylation by the thiol ester is significantly higher than that of the corresponding alkyl oxygen ester. Furthermore, the nitrophenyl ester reacts with the enzyme faster than the corresponding thiol ester.

Acylation of Carlsberg subtilisin by acetate and Z-glycinate esters. Since the relative rate of acylation of subtilisin by thiol and oxygen esters may depend on the specificity of the substrate, we studied the acylation reaction by using the derivatives of Z-glycine and acetic acid. Z-Glycine esters are fairly good substrates of subtilisin whereas the acetate esters are very poor ones.

The pK_a and k(limit) values were calculated from the pH-dependence of the reactions and are compiled in Table 2. It is seen that similarly to what is found

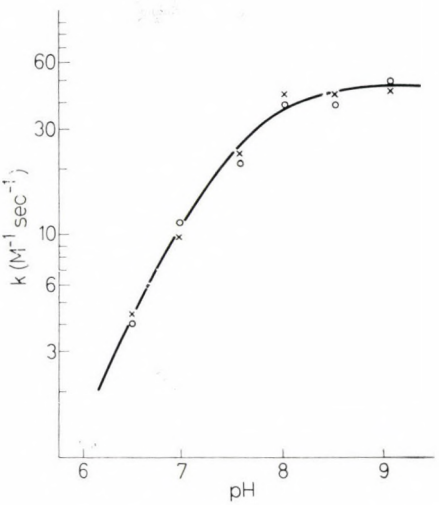


Fig. 1. pH-dependence of the reaction of ethyl thiolcinnamate with Carlsberg subtilisin. Reaction conditions are shown in the first part of Table 1. The experimental points were calculated from measurements at 310 and 250 nm. The solid line is a theoretical curve with parameters shown in Table 2

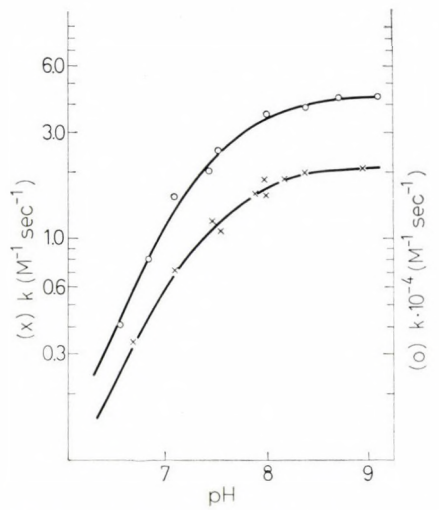


Fig. 2. pH-dependence of the hydrolysis of ethyl and p-nitrophenyl cinnamate by Carlsberg subtilisin. The reaction mixture for ethyl cinnamate (×) contained $2.0-10.2\times10^{-5}$ M enzyme and $4.5-40.8\times10^{-5}$ M substrate at 25.0°. In the case of p-nitrophenyl cinnamate (○) 6.0×10^{-6} M substrate reacted with 3.58×10^{-6} M enzyme at 25.0°. The solid lines are theoretical curves with parameters shown in Table 2

Table 2

*Kinetic parameters of reactions catalyzed by Carlsberg subtilisin**

Substrate	pK_a	k(limit) $M^{-1}\,sec^{-1}$
Ethyl acetate	—	0.064**
Ethyl thiolacetate	7.27	3.03
p-Nitrophenyl acetate***	7.05	2 100
Ethyl cinnamate	7.40	2.1
Ethyl thiolcinnamate	7.50	48.5
p-Nitrophenyl cinnamate	7.45	44 000
Ethyl Z-glycinate	7.20	308
Ethyl Z-thiolglycinate	7.18	25 600
p-Nitrophenyl Z-glycinate	7.20	1 730 000

* 25.0°, 1.7% acetonitrile
** Calculated for a pK_a of 7.20
*** cf. Polgár (1968)

with the cinnamoyl derivatives, the thiol esters also react in the case of acetyl and Z-glycyl derivatives at a higher rate than the corresponding oxygen esters. The reaction rates with the nitrophenyl esters are even higher.

Acylation of α-chymotrypsin with thiol and the corresponding oxygen esters. The acylation of chymotrypsin by p-nitrophenyl acetate and p-nitrophenyl thiolacetate proceeds at a similar rate (Frankfater, Kézdy, 1971). Therefore, it is important to establish whether chymotrypsin reacts similarly to subtilisin or not with alkyl ester and alkyl thiol ester substrates. The pH-dependence of the reaction of chymotrypsin with ethyl thiolcinnamate is presented in Fig. 3. In agreement with previous investigations on the acylation of chymotrypsin with other substrates (Bender et al., 1963), a bell-shaped pH-rate profile showing two pK_a values was obtained ($pK_1 = 7.20$, $pK_2 = 8.90$, calculated as proposed by Alberty and Massey, 1954). In this respect chymotrypsin differs from subtilisin, as the acylation of the latter shows a sigmoid pH-dependence. Another difference from subtilisin is the lower K_m of chymotrypsin, which renders it possible to determine k_{cat} and K_m values from Lineweaver-Burk plots. These kinetic parameters measured at pH 8.0 are shown in Table 3. The maximum of the bell-shaped curve is at about pH 8.0 where the rate constant does not change significantly with pH.

The rate constants of acylation by ethyl and p-nitrophenyl cinnamate are shown in Table 3. The rate constant of the reaction of ethyl cinnamate is similar to that found earlier for the reaction with methyl cinnamate (Bender, Zerner, 1962). However, the second-order rate constant of acylation with p-nitrophenyl cinnamate is much higher in 1.7% acetonitrile than in the presence of 10% acetonitrile (11.3×10^3 $M^{-1}sec^{-1}$, cf. Bender et al., 1962). This is in agreement with the observation that K_m becomes higher with increasing concentration of organic solvent (Applewhite et al., 1958; Faller, Sturtevant, 1966).

Similarly to what is found in the case of the cinnamoyl derivatives, chymotrypsin reacts with ethyl Z-thiolglycinate faster than with the corresponding oxygen ester derivative. Fig. 3 shows the bell-shaped pH-rate profile of the reaction of ethyl Z-thiolglycinate ($pK_1 = 6.94$, $pK_2 = 8.96$). The kinetic parameters of the reaction with the substrate and with ethyl and p-nitrophenyl Z-glycinate are compiled in Table 3.

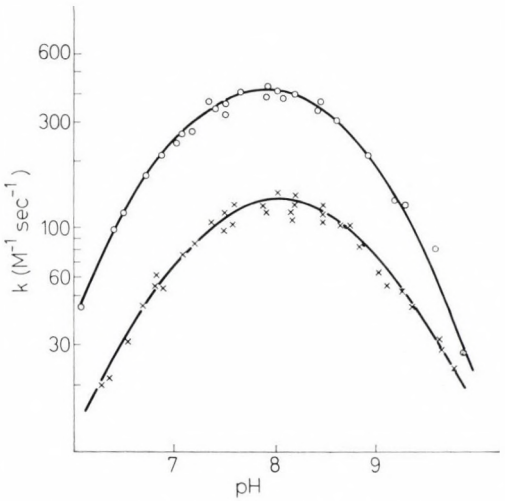


Fig. 3. pH-dependence of the hydrolysis of ethyl thiolcinnamate and ethyl Z-thiolglycinate by α-chymotrypsin. The reactions were measured at 25.0° in 0.1 M phosphate or carbonate buffer containing 1.7% acetonitrile. The reaction mixture for ethyl thiolcinnamate (×) contained 8.9×10^{-3} M substrate and $20.7-74.8\times10^{-6}$ M enzyme for ethyl Z-thiolglycinate (○) 11.2×10^{-5} M substrate and 1.24×10^{-5} M enzyme

Table 3

*Kinetic parameters of hydrolytic reactions catalyzed by α-chymotrypsin**

Substrate	k_{cat}/K_m M^{-1} sec^{-1}	$k_{cat}\times10^3$ sec^{-1}	$K_m\times10^6$ M
Ethyl cinnamate	3.65		
Ethyl thiolcinnamate	130	12.6	95
p-Nitrophenyl cinnamate	86 000**	9.65	0.112
Ethyl Z-glycinate	3.08		
Ethyl Z-thiolglycinate	420	274	660
p-Nitrophenyl Z-glycinate	241 500**	261	1.08
Ethyl N-acetyl L-phenylalaninate	54 000		
Ethyl N-acetyl DL-thiolphenylalaninate	1 000 000		

* 25°, pH 8.0, 1.7% acetonitrile
** Calculated from Lineweaver-Burk plot.

The most specific substrates of chymotrypsin are the aromatic amino acid derivatives. Therefore, we have also measured the rate constants for ethyl N-acetyl-thiolphenylalaninate and the corresponding oxygen ester (Table 3). Here, too acylation goes faster with thiol esters than with oxygen esters. It should be noted that a DL-thiol-ester was used for acylation. However, the D-enantiomer did not react under the conditions of the measurement and presumably did not interfere with the reaction of the specific L-enantiomer.

Deacylation of Carlsberg thiolsubtilisin

By using Carlsberg thiolsubtilisin pre-treated at room temperature for one hour with 0.05 mole equivalents of phenylmethanesulfonyl fluoride, the rate constants for the thiolenzyme can be properly determined.

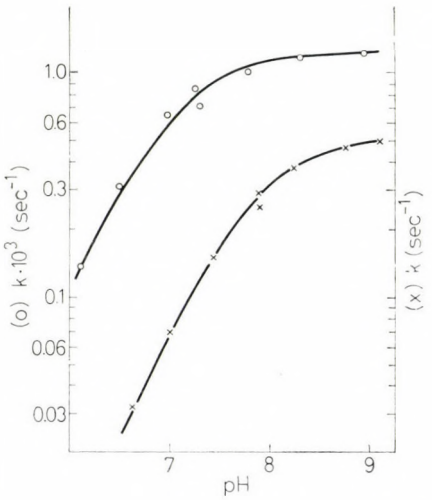


Fig. 4. pH-dependence of decinnamoylation of Carlsberg subtilisin and thiolsubtilisin. The hydrolysis was followed at 310 nm with cinnamoyl-subtilisin (×) and at 320 nm with cinnamoyl-thiolsubtilisin (○) at 25.0°. The concentration of the enzyme was about 6×10^{-6} M

Fig. 4 illustrates the pH-dependence of the hydrolysis of the cinnamoyl derivative of subtilisin and thiolsubtilisin. The pK_a of the single ionizable group on which the reaction depends is 7.8 for subtilisin and 7.0 for thiolsubtilisin. The pH-independent maximal values of the rate constants (k_3(limit)) are shown in Table 4. Similarly to the behavior of the derivatives of the Novo enzyme, the rate of deacylation of Carlsberg cinnamoyl-thiolsubtilisin is much lower than that of Carlsberg cinnamoyl-subtilisin.

The kinetic parameters of the hydrolysis of p-nitrophenyl acetate catalyzed by the Carlsberg thiolsubtilisin were determined as described previously for the Novo enzyme and are shown in Table 4. The rate constant of the overall reaction,

k_{cat}, refers to deacylation since the hydrolysis of the acetyl-enzyme is the rate-determining step.

It is seen from Table 4 that the rate of deacylation of Carlsberg thiolsubtilisin is much lower than that of the serine enzyme. The corresponding rate constants of the Novo subtilisin, also presented in Table 4, are similar to those of the Carlsberg enzyme.

Table 4

Kinetic constants (limit) of subtilisins[a]

	Novo		Carlsberg	
	OH	SH	OH	SH
$k_3 \times 10^2$ sec^{-1} of decinnamoylation	21[b]	0.043[b]	52[c]	0.125
$k_{cat} \times 10^2$ sec^{-1} of deacetylation with p-nitrophenyl acetate	35[b, d]	0.93[b]	110[c]	2.4
k_{cat}/K_m $M^{-1}sec^{-1}$ of acetylation with p-nitrophenyl acetate	680[b]	570[b]	2100[c]	360
k_{cat}/K_m $M^{-1}sec^{-1}$ of acetylation with ethyl thiolacetate	1.50	1.40	3.07	1.55

[a] 25.0°
[b] Data of Polgár, Bender (1967).
[c] Data of Polgár (1968).
[d] Corrected. (Based on titration with N-*trans*-cinnamoyl imidazole)

The reaction of thiolsubtilisin with ethyl thiolacetate

The studies on the symmetry of acylation and deacylation of serine proteases imply the question whether thiolsubtilisin is able to hydrolyze thiol esters. Previous investigations (Plogár, Bender, 1967) indicated that thiolsubtilisin only reacts with substrates containing good leaving groups, like nitrophenyl esters. In this respect mercaptanes are poorer leaving groups than nitrophenols but they are certainly better than alcohols. Ethyl thiolacetate was chosen as substrate for testing this problem since deacylation has already been demonstrated in this case (see Table 4).

The rate of acylation of thiolsubtilisin with ethyl thiolacetate is similar to that of the corresponding serine enzyme (Table 4). However, the pH-dependences of the two reactions are significantly different. Whereas the reaction with Carlsberg subtilisin depends on an ionizing group with a pK_a of 7.27 (Table 2), the acylation of the thiolenzyme is independent of pH between 6.2 and 8.0. This pH-independent rate constant is seen in Table 4. Similar results were obtained with Novo subtilisin. It should be emphasized that acylation by p-nitrophenyl acetate and alkylation by iodoacetamide are also independent of pH around neutrality (Polgár, Halász, 1971).

The alkaline hydrolyses of esters and thiol esters

The alkaline hydrolysis of esters is a simple model of the acylation of serine proteases where the hydroxyde ion is the nucleophile rather than the serine residue at the active site of the enzyme. Both reactions proceed through the formation of a tetrahedral intermediate. Accordingly, a comparison of the alkaline hydrolysis of oxygen and the corresponding thiol esters may lead to important conclusions as to the mechanism. Tarbell and Harnish (1951) have found that there is not too much difference between the alkaline hydrolysis rates of oxygen and thiol esters as measured in 62% aqueous acetone.

Table 5 shows the second-order rate constants of the alkaline hydrolysis of the substrates used in the above enzymatic reactions. The hydrolysis was performed in aqueous solution containing 1.7% acetonitrile as in the case of the enzymatic reactions. It is seen that thiol esters and the corresponding oxygen esters are hydrolyzed at comparable rates in agreement with the experiments performed in aqueous acetone. On the other hand, as it is expected (cf. Kirsch, Igelström, 1966) nitrophenyl esters react at a much higher rate due to the strong electron-withdrawing power of the nitrophenyl group.

Table 5

Rate constants of alkaline hydrolysis of esters

The reaction mixtures of 0.1 M ionic strength contained 1.7% acetonitrile at 25.0°. The rate constants are the average of 3—6 measurements in the pH-range indicated.

ster	Initial ester concentration $M \times 10^5$	pH-range	k_{OH^-} $M^{-1}\,sec^{-1}$	Method
Ethyl acetate	500	12.18—12.50	0.148	NH_2OH
Ethyl thiolacetate	33	11.65—12.46	0.155	250 nm
p-Nitrophenyl acetate	2.76	9.58—10.44	22.1	400 nm
Ethyl cinnamate	4.55	12.52—12.75	0.053	280 nm
Ethyl thiolcinnamate	2.10	12.40—12.60	0.033	310 nm
p-Nitrophenyl cinnamate	0.475	10.37—10.76	4.16	400 nm
Ethyl Z-glycinate	233	10.90—11.46	0.92	NH_2OH
Ethyl Z-thiolglycinate	33	10.95—11.38	1.58	250 nm
p-Nitrophenyl Z-glycinate	5.05	8.92— 9.81	290	400 nm

Discussion

The problem of symmetry in mechanism (repetition and nonrepetition) and in stereochemistry of the catalysis by serine proteases

The study of the reactions of thiol esters and thiolenzymes provides an experimental basis to treat the problem of symmetry of acylation and deacylation of serine proteases. Here the symmetry of the mechanism and the stereochemistry

of the two steps – i.e. the components of the rate-limiting transition state and their steric positions, respectively – will be discussed.

The mechanism of acylation and deacylation of chymotrypsin was earlier shown to be symmetrical with alkyl ester substrates (Bruice, 1961; Bender, 1962) that is indicated by the forward and reverse directions of equation (2), respectively. In other words, general base-catalyzed formation and general acid-catalyzed decomposition of the tetrahedral intermediate are repetitive in acylation and deacylation.

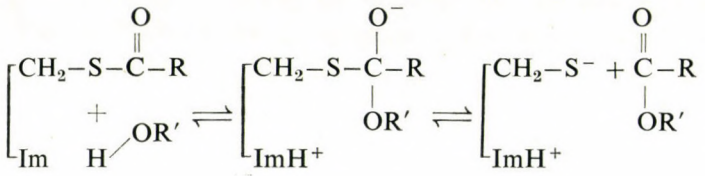
$$E + (S \text{ or } P) \underset{\text{general acid}}{\overset{\text{general base}}{\rightleftharpoons}} THI \underset{\text{general base}}{\overset{\text{general acid}}{\rightleftharpoons}} ES' \qquad (2)$$

On the other hand, on the basis of the good leaving property of the nitrophenolate ion, it was suggested that it departed from the tetrahedral intermediate without any assistance of general acid catalysis (Wang, 1968; Polgár, Bender, 1969a). This is supported by the data of Table 4, which shows that in the reaction of p-nitrophenyl acetate, deacylation is highly impaired relative to acylation if subtilisins are converted to thiolsubtilisins. The lack of symmetry can be observed also with ethyl thiolacetate (Table 4). This indicates that in the case of thiol esters, acylation proceeds *via* a simpler mechanism than does deacylation (nonrepetition). In fact, it was shown that in the hydrolysis of ethyl trifluorothiolacetate, the formation of the tetrahedral intermediate is a general base-catalyzed process but its breakdown does not require general acid catalysis (Fedor, Bruice, 1965), in contrast to what is found for the hydrolysis of the corresponding oxygen ester (Bender, Heck, 1967).

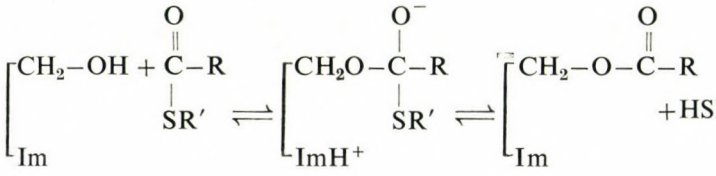
Accordingly, the nature of the leaving group is an important factor of the mechanism. It may be noted that in the case of anilide and peptide substrates the mechanisms of acylation and deacylation are symmetrical if general base and general acid catalyses are considered as criteria, although the rate-determining step is different in acylation and deacylation: the breakdown and the formation of the tetrahedral intermediate, respectively (Polgár, Bender, 1969; Caplow, 1969).

It is obvious from the foregoing that a lack of symmetry of the mechanism can be expected if different chemical bonds are broken in acylation and deacylation. In the hydrolysis of acyl-thiolsubtilisins (equation 3) and in the reaction of subtilisin with thiol esters (equation 4), the same chemical bond is broken in deacylation and acylation. The chemistry of the two reactions is identical: general base-catalyzed attack of an oxygen nucleophile on a thiol ester bond. Thus the mechanism of the two reactions represented by equations (3) and (4), should be identical. However, stereochemical features, i.e. the orientation, rigidity and environment of the reacting groups are different. That oxygen is substituted for a sulfur atom on the enzymatic side and on the solvent side of the tetrahedral intermediate is shown in equations (3) and (4), respectively. Similarly, the hydroxyl group of water and the serine residue attacks the carbonyl carbon atom from the solvent and enzymatic side, respectively. The comparison of Tables 4 and 2 shows that the replacement of oxygen by sulfur atom on the enzymatic side of the tetrahedral

intermediate results in a great decrease in enzymatic activity whereas the same change on the solvent side does not impair but rather enhances the reactivity. This indicates that the solvent side of the tetrahedral intermediate, in contrast to the enzymatic side, is not sensitive to minute changes in the geometry of the reacting groups.



(3)



(4)

Orientation and rigidity

The distortion of the active site by substituting sulfur for the oxygen atom influences the orientation of the reacting groups. Opinions are different as to the importance of orientation (Storm, Koshland, 1970; Bruice et al., 1971; Page, Jencks, 1971). We would like to emphasize that controversity necessarily emerges if orientation is considered alone and rigidity is disregarded. It is difficult to separate the contribution of these two factors to the reaction rate since systems of different orientation may have different rigidity. Indeed, the hydrolysis of acyl-thiolsubtilisins suggests a close connection between orientation and rigidity as follows. The active site together with the acyl-moiety of a *specific* substrate forms a rigid system. Any distortion which leads to improper orientation of the reacting groups is preserved in the rigid system resulting in the inactivation of the enzyme. In a less rigid system, such as formed with a *nonspecific* substrate, the substrate has more freedom of motion and takes different positions, some of which are suitable for reaction. In fact, we have shown the O → S replacement impaired the deacylation of acetyl-thiolsubtilisin much less than that of Z-glycyl-thiolsubtilisin which contains a more specific acyl-moiety (Polgár, 1968; Polgár, Bender, 1969b).

It may be concluded that the enzymatic side of the tetrahedral intermediate is more rigid and the solvent side is more flexible. Therefore, a distortion at the enzymatic side (change from serine protease to thiolenzyme) results in a decrease in the rate of the hydrolysis of the acyl-enzyme even though the mechanism is simplified. On the other hand, the distorted orientation at the solvent side (acylation by thiol esters) can be compensated for by the higher freedom of motion.

The nature of the tetrahedral addition intermediate

Tables 2 and 3 clearly show that subtilisin and chymotrypsin, respectively, react with alkyl thiol esters at a much higher rate than with the corresponding oxygen esters. This is equally valid for the nonspecific ethyl thiolacetate and for the most specific ethyl N-acetyl-thiolphenylalaninate. On the other hand, it is seen from Table 5 that there is no appreciable difference between the rates of the alkaline hydrolysis of oxygen esters and the corresponding thiol esters.

The alkaline hydrolysis of esters and thiol esters proceeds through the formation of a tetrahedral addition intermediate, and it was proposed that a similar tetrahedral intermediate was formed in the enzymatic hydrolysis, too (Bender, Kézdy, 1965). Of course, the alkaline hydrolysis can be regarded as a model of the enzymatic process. In both cases the attack of the oxygen nucleophile is the rate-limiting step. However, the different behavior of esters and thiol esters in enzymatic and alkaline hydrolysis indicates that the formation of the intermediate, the rate-limiting step, is somehow different in the enzymatic and in the model reaction. This is in agreement with the one-encounter mechanism proposed for serine proteases where the tetrahedral intermediate is stabilized by an extended hydrogen-bond network (Polgár, 1972).

In a recent work, Frankfater and Kézdy have shown that p-nitrophenyl acetate and p-nitrophenyl thiolacetate are hydrolyzed by chymotrypsin and by the hydroxide ion at similar rates (Frankfater, Kézdy, 1971). From this finding they have concluded that a similar tetrahedral intermediate is formed in the enzymatic and in the alkaline hydrolysis. This is apparently at variance with our finding that the enzymatic and the alkaline intermediates are different. However, the contradiction is reconciled if one considers that the detailed enzymatic mechanism may be different for substrates of different type.

Acylation of chymotrypsin with p-nitrophenyl acetate and p-nitrophenyl thiolacetate occurs *via* a minimal mechanism involving only a general base-catalyzed attack by the serine residue on the carbonyl carbon atom of the substrate. This is very similar to the attack by the hydroxide ion. Thus the tetrahedral intermediates, formed in the enzymatic and alkaline hydrolyses of p-nitrophenyl acetate and p-nitrophenyl thiolacetate are expected to be similar, which is indeed the case as shown experimentally (Frankfater, Kézdy, 1971), and by the similar Hammett ρ-constants for enzymatic and alkaline hydrolyses of phenyl acetates (Bender, Nakamura, 1962).

Finally, I would like to point out that the detailed mechanism of action of serine proteases may depend not only on the chemical nature of the leaving group but also on the specificity of the substrate which is determined by the mode of binding. In the case of specific substrates, electrophilic assistance on the carbonyl oxygen atom is added to general base–general acid catalysis (Polgár, 1972; Williams, 1970). This is consistent with the fact that lower Hammett ρ-constants were found for acylation by specific substrates than for acylation with phenyl acetate (Williams, 1970; Williams, Bender, 1971). Accordingly, electrophilic assistance may be important with a specific nitrophenyl ester but not with nitrophenyl acetate. This is

supported by the observation that in the reaction of chymotrypsin with p-nitrophenyl acetate, the leaving group rather than the acyl moiety binds to the specificity site (Bender et al., 1964). Such a binding provides favorable geometry only for the nucleophile attack on the carbonyl carbon atom but not for the electrophilic assistance on the carbonyl oxygen atom.

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Effect of Chemical Mediators on the K^+-Efflux, Ca^{2+}-Uptake and ^{32}P-Incorporation of Erythrocytes

(Preliminary Communication)

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Gárdos (1958) described the phenomenon of Ca^{2+}-dependent rapid K^+-efflux from erythrocytes that can be evoked whenever cellular ATP decreases to a minimum. This degree of ATP depletion can be achieved within 1 hour if cells are incubated in the presence of IA + inosine at 37° C (Gárdos, 1956). We reported previously that the Ca^{2+}-dependent rapid K^+-efflux can be accelerated by histamine (Gárdos, Szász, 1968), serotonin and norepinephrine (Szász, Gárdos, 1971). Since all these biogenic amines are known to be activators of adenyl cyclase, we tested also theophylline, the well-known cyclic nucleotide phosphodiesterase inhibitor, which ought to affect the phenomenon in a similar way, if cAMP is involved in its development. On the other hand, from among the mediator biogenic amines, acetylcholine – being not linked with cAMP – should behave differently. Fig. 1 shows that these expectations could be justified experimentally. A more direct proof was also obtained by adding the permeable dibutyryl-cAMP to erythrocytes in a concentration range that is effective in the intact brain tissue. Dibutyryl-cAMP accelerated K^+-efflux in a strictly Ca^{2+}-dependent way (Fig. 1).

In order to eliminate the possible disturbing effects of IA-membrane interactions we reproduced the process even with erythrocytes depleted from 2,3-DPG and ATP, respectively, by 15 mM $NaHSO_3$ (5 hours at 37° C) and 8 mM NaF + + 2.5 mM Na_2HAsO_4 (12 hours at 37° C) treatment. All inhibitors were washed off in a K^+-rich medium to avoid the loss of cellular K^+. ATP level of these cells was less than 10^{-8} moles/l erythrocytes as shown by the firefly lantern method, and no ATP regeneration was observed during incubation at 37° C. Rapid K^+-efflux could be evoked in these cells by adding $CaCl_2$ without any inhibitor. In these ATP-depleted cells, however, histamine and theophylline did not accelerate K^+-efflux either in the presence or in the absence of IA, indicating that for the development of the permeability effects of these drugs at least a small amount of cellular ATP is necessary.

We intended to check whether the Ca^{2+}-dependence of the K^+-efflux was due to the incorporation of Ca^{2+} into the membrane of the ATP depleted erythrocytes or not. To this end we added $^{45}CaCl_2$ to the incubation media. The ^{45}Ca

* Abbreviations: ATP, adenosine 5'-triphosphate; cAMP, adenosine 3',5'-cyclic monophosphate; 2,3-DPG, 2,3-diphosphoglyceric acid; EGTA, ethylenglycol bis (β-aminoethylether)N,N'-tetraacetate; IA, iodoacetate.

influx patterns (Fig. 2) were similar to the changes found in K^+-efflux. However, ^{45}Ca taken up by the cells was distributed so that its majority was found in the acid soluble fraction. Gel filtration of the hemolysate through a Sephadex G−25 column also yielded only traces of isotope in the membrane and hemoglobin fraction, and the great majority was eluted with the small molecular weight fraction. We could not reliably measure the trace amounts of ^{45}Ca of the membrane fraction. Gent et al. (1964), however, established the relationship between the

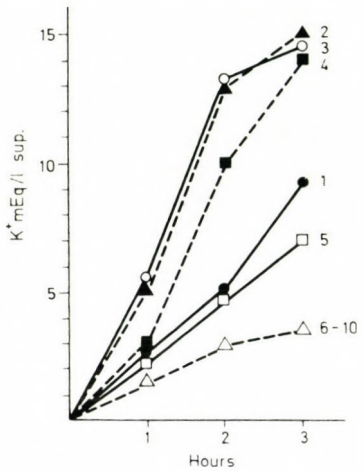

Fig. 1. Ca^{2+}-dependent rapid K^+-efflux from human erythrocytes. The systems contained: 2.5 mM IA, 10 mM inosine, 2.5 mM $CaCl_2$. Additions: 1: None; 2: 0.5 mM histamine; 3: 0.5 mM theophylline; 4: 0.5 mM dibutyryl-cAMP; 5: 1 mM acetylcholine, 0.15 mM neostigmine; 6: 2.5 mM EGTA; 7: 0.5 mM histamine, 2.5 mM EGTA; 8: 0.5 mM theophylline, 2.5 mM EGTA; 9: 0.5 mM dibutyryl-cAMP, 2.5 mM EGTA; 10: 1 mM acetylcholine, 0.15 mM neostigmine, 2.5 mM EGTA. Incubation temperature: 37° C. Haematocrit: 30

Ca^{2+} concentration of the medium and the erythrocyte membrane, being

$$\frac{x}{x_{max}} = \frac{km}{1 \pm km}$$

where x is the membrane-bound Ca^{2+}, m the Ca^{2+} concentration of the medium, and k a constant. This correlation is most probably valid in our case, too.

It is to be mentioned that ^{45}Ca influx was enhanced by histamine and theophylline even in perfectly 2,3-DPG and ATP-depleted cells, although the K^+-permeability of these cells was unaffected by these drugs. This finding is to be considered when we search for the first step in the series of events induced by compounds acting through cAMP. These results might draw the attention to the primary importance of altered Ca^{2+} permeability. Ca^{2+}-chelation – described in case of theophylline (Chenoweth, 1956) – is to be kept in mind as a possible mechanism of the enhanced Ca^{2+}-influx.

cAMP is known to activate phospholipid kinases and protein kinases (Rasmussen, Tenenhouse, 1968). This way the main membrane components could be phosphorylated by ATP and could become Ca^{2+}-acceptors. Acetylcholine, on the other hand, is known to activate dephosphorylation processes (Durell at al., 1969). In order to study the phosphorylated state of the membrane, $AT^{32}P$ was synthesized in erythrocytes by adding $^{32}P_i$ + inosine + adenine to the cells (Szász,

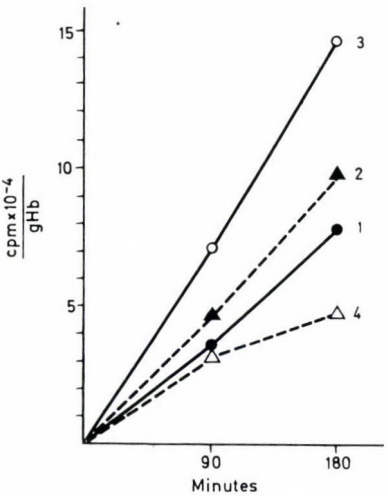

Fig. 2. ^{45}Ca radioactivity in hemolysates. Erythrocytes were incubated with 2.5 mM IA and 10 mM inosine; 100 μCi ^{45}Ca/ml erythrocyte were added (specific activity: 1.86 Ci/g Ca). Additions: 1: None, 2: 0.5 mM histamine; 3: 0.5 mM theophylline; 4: 1 mM acetylcholine, 0.15 mM neostigmine. Incubation temperature: 37° C. Haematocrit: 30. After incubation cells were washed 5 times with 0.16 M NaCl. Sediments were hemolysed with equal volumes of 0.1 % saponin. The radioactivity of 0.05 ml decolorized aliquots was measured in a Packard Tri-Carb scintillation spectrometer (1 nmole = 6700 cpm)

1970). The pretreated cells were mixed with the reagents and the radioactivity of the acid-insoluble fraction was measured (Fig. 3). The radioactivity of this fraction could easily be detected and could be attributed with high probability to the phosphorylation of the membrane. The changes are in concord with the K^+- and Ca^{2+}-permeability data in fresh erythrocytes treated with IA + inosine. In 2,3-DPG and ATP-depleted cells, labelled only with $^{32}P_i$ (i.e. in $AT^{32}P$ free cells) the differences are insignificant, and that is the case if lipids are extracted from the $AT^{32}P$-labelled cells treated previously as described above. Further experiments are needed, however, to prove the eventual role of ATP-phosphorylated membrane phospholipids in the Gárdos-phenomenon.

All the above findings could be explained by the cAMP theory as follows. The enzymes activated by cAMP would phosphorylate the membrane constituents with the aid of ATP. These phosphate acceptors could bind Ca^{2+}, if a high intracellular ATP concentration does not compete for Ca^{2+}. In case these membrane

acceptors interact with Ca^{2+}, the membrane structure produced will favour K^+-efflux. The main difficulty in this interpretation is the doubtful existence of the enzymes synthesizing and destroying cAMP in the mature enucleated erythrocytes. The adenyl cyclase activity in rat erythrocytes has recently been ascribed by some authors (Quiring et al., 1972) to reticulocyte contamination. On the other hand, as for the enzyme 2'-3'-cyclic nucleotide 3'-phosphohydrolase, recent accurate measurements proved its existence in mature human erythrocytes (Sudo et al., 1972). We did not try to demonstrate adenyl cyclase or cyclic nucleotide phosphodiesterase activities in the cells. We separated the erythrocytes from the granulo-

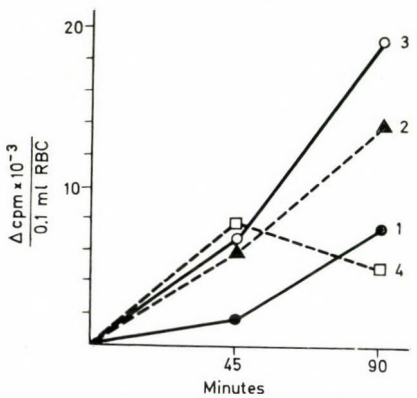

Fig. 3. ^{32}P-incorporation into the membrane of erythrocytes containing $AT^{32}P$. Preincubation medium: 10 mM inosine, 1 mM adenine, 3 mM Na phosphate (pH 7.4); 250 μCi carrier free $Na_2H^{32}PO_4$ was added per ml erythrocyte. Preincubation temperature: 37° C. Preincubation time: 90 min. Cells were washed 6 times in 10 volumes of 0.16 M NaCl containing 5 mM $MgCl_2$ and 5 mM glucose. Incubation temperature: 37° C. The systems contained: 2.5 mM IA, 10 mM inosine, 2 5 mM $CaCl_2$. For additions see Fig. 2. Samples were precipitated with equal volumes of 20% trichloroacetic acid and washed three times with 10% trichloroacetic acid. Sediments were hydrolyzed in 33% KOH, neutralized and the radioactivity of 0.2 ml decolorized aliquots was measured on the basis of Čerenkov-effect in a Packard Tri-Carb scintillation spectrometer (1 pmole = 2800 cpm)

cytes and platelets by selective adsorption (Langfelder, Zsdánszky, 1966) and separated the young and old erythrocyte populations by centrifugation. By testing these fractions it could be clearly shown that the Gárdos-effect manifested itself in purified old erythrocyte populations, too, and these cells were affected by histamine and theophylline. Many further questions are open as to the explanation of the mechanism of the permeability phenomenon studied: e. g. the ineffectiveness of epinephrine found by us, as well as the Ca^{2+}-dependent increase in K^+ permeability induced by the β-adrenergic agent isoproterenol (Szász, unpublished results) and the β-adrenergic receptor-blocking agent propranolol (Manninen, 1970), etc. Our further experiments are aimed at the elucidation of these problems.

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Water Structure and Microwave Ion Mobility in Gelatine Solutions

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Ionic conductivity of KCl, NaCl and $CaCl_2$ was measured in 10, 20 and 30 per cent gelatine solutions at 2.6 GHz frequency. With the increase of gelatine concentration conductivity decreases to a degree higher than expected on the basis of the obstruction effect. If we consider that the mobility of ions decreases in inverse ratio to the increase in time of the dielectric relaxation indicative of the bound state of the water phase, the measured and the calculated values of conductivity agree well in solutions containing NaCl and KCl. In solutions containing $CaCl_2$ also ion-binding greatly contributes to the decrease of ionic conductivity beside the decreased ion mobility.

Introduction

The conductivity of the intracellular space called "inner conductivity", is 2.5 to 3.0 times lower in erythrocytes and muscle than the value measured in the ash-solution of the incinerated tissue or the value calculated on the basis of the ion composition of the inorganic substance (Ernst, 1963).

The low conductivity of tissues is a consequence partly of the ion-binding of cell proteins, and partly that of the decreased ion mobility. According to Pauly and Schwan (1966) the degree of the decrease of ion mobility is higher than expected on the basis of the effect exerted by the haemoglobin molecule on the distribution of electric field (the obstruction effect).

According to other data (Ernst, 1963; Pócsik, 1969; Hazlewood et al., 1969; Cope, 1969; Masszi, 1972) the water phase is characterized by a certain degree of boundness both in the cell plasma and the polyelectrolyte solutions modelling the plasma. In connection with these experimental results the question is necessarily raised what significance has to be ascribed to the change of the state of water in the decrease of ion mobility.

In the present experiments the conductivity of ions was measured in gelatine solutions at 2.6 GHz frequency, and the measured values were compared with the dielectric relaxation time of the water phase, a data changing in inverse ratio to the viscosity of the water phase and so also to ion mobility.

* With the technical assistance of Miss Judith Gátonyi.

Material and method

Conductivity measurements were based on the measurement of the microwave damping factor (α). A detailed description of the method was published in a previous paper (Masszi, Örkényi, 1967; Masszi, 1970). The difference between the damping factor of the solutions containing and non-containing the ion in question, i.e. the $\Delta\alpha$ value, was determined. The conductivity corresponding to the ion content (σ) was determined on the basis of this value by using the equation

$$\sigma = \frac{\sqrt{\varepsilon'} \cdot \Delta\alpha}{60 \cdot \pi} \; [\text{ohm}^{-1}\,\text{cm}^{-1}] \qquad (1)$$

where ε' is the microwave dielectric constant of the solution. It was possible to formulate the equation in a simple form because the relatively low ion concentration used in the measurements decreases the dielectric constant of the solution with less than 3 per cent. So, when the equation was deduced, the dielectric constants of the two solutions were considered to be the same, i.e. the dielectric constant of the solution not containing ion was used in both cases (Masszi, Örkényi, 1969a).

The low-frequency conductivity of different solutions was determined at 20 kHz in a measuring cell used for microwave measurements with an RC-bridge method. In gelatine solutions the conductivity corresponding to the ion content in question – similarly to microwave measurements – was provided by the difference between the conductivity of the solution containing ion and the conductivity of the solution not containing ion.

Gelatine solutions containing 3 mg ash per one gram of dry substance were used in the measurements. The gelatine was previously dehydrated by drying at 60°C for 24 hours. Then it was dissolved in distilled water or in a solution of 0.1 normal concentration until the desired concentration was reached.

The dry weight content was controlled by drying at 110°C. We were able to adjust the concentration of dry substance with an accuracy of $\pm$ 1 per cent. The conductivity of the water used for dilution was lower than 10^{-4} ohm^{-1} cm^{-1}.

Results

1. It was pointed out previously that the low-frequency conductivity and the microwave conductivity of electrolyte solutions do not differ significantly, but the ionic conductivity measured by microwave in polyelectrolyte solutions shows a 10 to 20 per cent increase as compared with the low-frequency values (Masszi, Örkényi, 1967). Table 1 compares the low-frequency and microwave values of ionic conductivity corresponding to 0.1 n NaCl content of a 20 per cent gelatine solution in a temperature range from 20 to 50°C. According to the data of the Table the microwave conductivity is, on an average, 17 per cent higher than the one measured at low frequency (20 kHz). According to the radio-fre-

quency measurements of Sachs et al. (1969) an increase of conductivity can be observed in polyelectrolytes in the MHz range, which is the result of the relaxation of the ion cloud. Thus microwave conductivity measurement has the advantage that the restraining effect connected with the deformation of the ion cloud has no significance here, and so it provides a more direct information about the connection of water structure and ion mobility.

Table 1

Comparison of microwave (σ) and low frequency (σ_l) conductivity

T C°	σ[mScm^{-1}]	σ_e[mScm^{-1}]	$\frac{\sigma}{\sigma_e}$
20	4.85	4.20	1.15
25	5.55	4.57	1.21
30	5.90	5.12	1.16
35	6.30	5.45	1.16
40	6.80	5.80	1.17
45	7.35	6.30	1.17
50	8.15	6.90	1.18
		Average:	1.17

2. Tables 2, 3 and 4 summarize the data of measurements of solutions containing 0.1 n KCl, NaCl and $CaCl_2$, respectively. The 2nd columns of the Tables contain the conductivity of aqueous solutions (σ_0). The further columns contain the values of relative conductivity compared with the conductivity of aqueous solutions (σ/σ_0). It is apparent that the relative conductivity rapidly decreases with increasing gelatine concentration. In gelatine solutions containing 10 and 20 per cent NaCl and KCl at the temperatue of the sol → gel transition, i.e. between 30 and 35°C, a slight increase of relative conductivity can be observed.

Table 2

Conductivity of KCl-containing solutions at temperatures of 20 to 50°C

T C°	σ_0 10^{-3}[ohm^{-1}cm^{-1}]	σ/σ_0		
		10 per cent	20 per cent	30 per cent
20	12.1	0.85	0.57	0.32
25	13.3	0.80	0.58	0.32
30	14.6	0.83	0.58	0.34
35	16.0	0.83	0.57	0.34
40	17.6	0.80	0.53	0.34
45	18.8	0.80	0.55	0.34
50	20.2	0.77	0.51	0.34
	Average:	0.81	0.56	0.33

Table 3

Conductivity of NaCl-containing solutions at temperatures of 20 to 50°C

T C°	σ_0 10^{-3}[ohm^{-1}cm^{-1}]	σ/σ_0		
		10 per cent	20 per cent	30 per cent
20	8.05	0.81	0.60	0.35
25	8.90	0.80	0.58	0.37
30	10.05	0.82	0.58	0.35
35	10.9	0.80	0.59	0.36
40	12.1	0.78	0.54	0.32
45	13.3	0.78	0.55	0.34
50	14.5	0.79	0.56	0.38
	Average:	0.80	0.57	0.35

Table 4

Conductivity of $CaCl_2$-containing solutions at temperatures of 20 to 50°C

T C°	σ_0 10^{-3}[ohm^{-1}cm^{-1}]	σ/σ_0		
		10 per cent	20 per cent	30 per cen
20	9.b	0.58	0.32	0.26
30	11.4	0.63	0.32	0.25
40	13.7	0.60	0.39	0.22
50	16.5	0.59	0.33	0.21
	Average:	0.60	0.34	0.23

Discussion

Robinson and Stokes (1959) examined the effect on ionic conductivity of non-electrolyte molecules (large ones when compared with electrolytes). They approximated the problem in two ways:

1. On the one hand, they pointed out that the results of the obstruction theory – which considers the effect of non-electrolyte on the distribution of electric field as an insulator inhomogeneity – can be expressed in the case of not too concentrated solutions in the following simple form:

$$\frac{\sigma}{\sigma_0} = 1 - kp\,, \tag{2}$$

where p is the volume proportion of the non-electrolyte, k is a form-factor connected with the form of non-electrolyte, the values of which are known from the Tables of Fricke (1924).

But, according to our measurements made with 10 and 20 per cent sucrose, mannite and glycerol solutions, the ionic conductivity decreases with decreasing concentration significantly stronger than it could be expected on the basis of equation (2).

The data in the literature show that the obstruction theory can be well applied for the case of suspended particles of microscopic size. For instance, it describes well the correlation between the volume of erythrocytes and the conductivity of the plasma in the case of whole blood (Pauly, Schwan, 1966), or the correlation between the conductivity of extracellular space and the volume ratio of intracellular space in muscle (Fatt, 1964). However, it cannot be applied for suspension of particles of colloid magnitude as is e.g. a haemoglobin solution (Pauly, Schwan, 1966). These data also indicate that, with the increase of the specific surface of the solute particles, the structure of water will be influenced by the interaction between the surface and water, an event leading beyond the obstruction effect, to a decreased ion mobility.

2. On the other hand, Robinson and Stokes determined the viscosity of the solution, and, in accordance with the Walden-Piszarzsevszkij empiric relation, they examined whether the conductivity really changed in inverse ratio to viscosity. According to their data the conductivity does not decrease as strongly as the viscosity of the solution increases. Our measurements performed in gelatine solutions show that the viscosity of the mixture is not appropriate for the examination of ion mobility for in sol → gel transition, when the solution passes over from a liquid to a solid state, the relative conductivity does not decrease correspondingly, in fact, it shows a slight increase.

It is an evident assumption that the ion mobility is not determined by the viscosity of the solution but by the viscosity of the water phase, in which the ion migration happens. In a previous paper we pointed out that the time of relaxation of the dipole rotation of the water gradually increases with increasing gelatine concentration. According to the Debye (1929) equation the time of relaxation changes proportionally to the viscosity of the water, η:

$$\tau = \frac{4\pi \cdot a^3}{kT} \eta, \tag{3}$$

where a is the radius of the water molecule, k is the Boltzmann constant, T is the absolute temperature. Equation 3 was proved also experimentally for water and heavy water at 20 to 50°C by Collie et al. (1948).

3. On the basis of equation 3 and of the Walden-Piszarzsevszkij formula it can be assumed as first approximation that in the water phase ionic conductivity decreases in τ/τ_0 ratio, where τ is the time of relaxation of the water phase in the gelatine solution in question, τ_0 is the time of relaxation in free water. Taking equation (2) into consideration the theoretically expectable relative conductivity is:

$$\frac{\sigma}{\sigma_0} \text{ theor.} = (1 - kp)\frac{\tau_0}{\tau} \tag{4}$$

The first line of Table 5 contains the $1 - kp$ factor, where $k = 1.7$ in thread-like gelatine molecules, $p = 0.68 \cdot c$; c is the percentage concentration of gelatine, 0.68 ml/g is the specific volume of gelatine (Stuart, 1953). The second line contains the τ/τ_0 values, which were determined in our previous work (Masszi, 1972).

Table 5

Comparison of theoretical and measured relative conductivities calculated on the basis of Equation 4

	10 per cent	20 per cent	30 per cent
$1-1.7 \cdot 0.68\,c$	0.89	0.77	0.65
$\frac{\tau_0}{\tau}$	0.89	0.72	0.64
$\frac{\sigma}{\sigma_0}$ theor.	0.79	0.51	0.39
$\frac{\sigma}{\sigma_0}$ exp. KCl	0.77	0.51	0.34
$\frac{\sigma}{\sigma_0}$ exp. NaCl	0.79	0.56	0.38
$\frac{\sigma}{\sigma_0}$ exp. $CaCl_2$	0.59	0.33	0.21

The third line contains, according to equation (4) the product of the two factors, the values of which agree well with the measured values in solutions containing KCl and NaCl.

According to our data the dependence on concentration of the ionic conductivity can be described by considering the obstruction effect and the time of relaxation. According to our calculation the role of ion-binding in the decrease of conductivity is not significant in solutions containing KCl and NaCl. This corresponds to our previous data according to which the energy of activation of the ionic conductivity of KCl in gelatine medium is equal to the value measured in water (Masszi, Örkényi, 1967a). Ling ang Cope (1969) demonstrated with NMR method that 20 per cent gelatine does not bind Na^+ ions. In solutions containing 20 to 30 per cent $CaCl_2$ the ratio of the calculated and measured values reveals that about 40 per cent of the Ca^{++} ions is in bound state.

In connection with the relation of the time of relaxation and ion mobility it would be reasonable to take into consideration the specific effect of different kinds of ions on the time of relaxation of water, but – as it is shown by the measurements of Giese et al. (1970) – the time of relaxation of the dipole rotation of water in 0.1 normal solutions decreases by about 1 per cent while the difference between different kinds of ions is of about 0.1 per cent magnitude which can be neglected in the first approximation.

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Dielectric Relaxation and Water Structure in Gelatine Solutions

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Microwave conductivity and dielectric constant of 5 to 50 per cent gelatine solutions were measured over a frequency range of 2.6 to 4.0 GHz. The average time of relaxation of the dipole rotation of water, which increases significantly with increasing gelatine concentration, was determined on the basis of the frequency-dependence of the microwave conductivity. From our data it can be concluded that water binding includes not only the water layer being in direct interaction with the protein, but the mobility of the water molecules decreases also in the whole water phase which is in connection with the decrease of the entropy of rotation of water. According to our data a further range of relaxation can be observed at dm wavelength, which presumably is connected with the dielectric relaxation of the water absorbed in the first layer.

Introduction

The high-frequency and microwave investigations of the recent years unambiguously prove that the frequency of relaxation of the so-called "water absorbed in the first layer" having interaction with the polarized groups of polyelectrolyte in polyelectrolyte solutions is about two orders of magnitude lower than that of the free water (Schwan, 1965; Grant, 1965; Masszi, Örkényi, 1967a; Pennock, Schwan, 1969; Hoekstra, Doyle, 1971). These results indicate that the structure of bound water differs from the structure of the so-called "bulk" water, but they contradict the "ice-berg" hypothesis because the frequency of relaxation of ice is 5 orders of magnitude lower than that of the bound water (Auty, Cole, 1952). It is a further question whether the water phase can be resolved to bound and the so-called "bulk" water, which is of the same structure as is common water and has thus the same physical and chemical characteristics, or, the more bound structure of water can be demonstrated also beyond the first layer. From vapour-pressure measurements, performed on gelatine gel and frog muscle, we can conclude that free and bound water cannot be sharply discriminated from one another (Ernst et al., 1950; Ernst, 1970). According to Ling water is bound to the polyelectrolytes to a gradually decreasing degree not only in the first layer but also in the other ones. He thinks this bound state spreading out to the whole water phase to have fundamental significance in understanding biological processes (Ling, 1965).

* With the technical assistance of Miss Judith Gátonyi.

This problem was experimentally examined with the aid of microwave measurements performed in 5 to 50 per cent gelatine solutions starting from the assumption as follows. From microwave dielectric parameters we can selectively determine the time of relaxation of the dipole rotation of the water which – according to the investigations already mentioned – can change very sensitively, by orders of magnitude, depending on the boundness of water.

Material and method

The microwave refractivity (n) and damping factor (α) of the materials under examination were determined with a method described in detail in a previous paper (Masszi, Örkényi, 1967; Masszi, 1970). The dielectric constant (ε') was determined from the measured parametres according to the equation

$$\varepsilon' = n^2 - \left(\frac{\lambda_0}{2\pi}\alpha\right)^2, \tag{1}$$

where λ_0 is the wavelength in vacuum. The microwave conductivity of the sample was determined on the basis of the equation

$$\sigma = \frac{\sqrt{\varepsilon'}\,\alpha}{60\pi}\ [\text{ohm}^{-1}\,\text{cm}^{-1}]. \tag{2}$$

The average wavelength of relaxation was determined with the aid of the equation

$$\sigma = \frac{\Delta\varepsilon' \cdot \bar{\lambda}_s}{54} \cdot f^2 + \sigma_{\text{om}}\ [\text{m ohm}^{-1}\,\text{cm}^{-1}], \tag{3}$$

where $\Delta\varepsilon'$ is the difference between the dielectric constants measured at low and high frequencies as compared with the frequency of relaxation. The low-frequency dielectric constant was substituted by the value measured at 2.6 GHz frequency, which is one order of magnitude lower than the 35 GHz frequency relaxation of water. The high-frequency dielectric constant was considered with the substitution $\varepsilon_\infty = 5$ on the basis of the data of Collie et al. (1948) measured in a wide range of frequency.

$\bar{\lambda}_s$ is the average wavelength of relaxation of water measured in cm.

f is the frequency measured in GHz

σ_{om} is the ohmic conductivity or conductivity originating in a process of relaxation appearing at lower frequency.

The detailed deduction of equation (3) is presented in the Appendix.

The measurements were performed at 2.63, 3, 3.5 and 4 GHz frequency. The temperature of the measuring cell was stabilized at the required level with an accuracy of 0.1°C. The conductivity of the different solutions was determined at 20 kHz with the Wheatstone-bridge method.

For the measurements we used gelatine powder of 3 mg/g ash content, which was dehydrated by drying at +60°C for 24 hours and then diluted with distilled water to the required concentration.

Results

Fig. 1 shows the frequency-dependence of the conductivity of 5 to 50 per cent gelatine solutions. The square of the frequency is shown by the abscissa and so – corresponding to equation (3) – the conductivity values belonging to

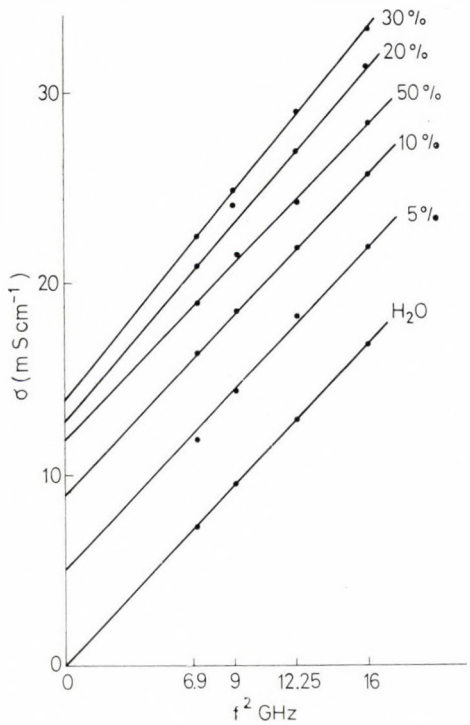

Fig. 1. Frequency-dependence of microwave conductivity of distilled water and of 5 to 50 per cent gelatine solutions at 50°C

the different solutions were placed along a straight line. Since the damping effect of water at 50°C is of so a slight degree that it cannot be measured with our method, the values concerning distilled water were taken from the review of Hasted (1961).

A straight line was fitted to the points of measurements of the different solutions according to the method of the smallest square difference, and, in the knowledge of $\Delta\varepsilon'$ the values of $\bar{\lambda}_s$ were determined from the slope of the straight line on the basis of equation (3). Moreover, the standard deviation of $\bar{\lambda}_s$ values

Table 1

Wavelength of relaxation ($\bar{\lambda}_s$) and time of relaxation (τ) of the dipole rotation of water in 0 to 50 per cent gelatine solutions at 50°C. (ε' is the dielectric constant of gelatine solutions at 2.6 GHz frequency)

Conc. per cent	ε'	$\bar{\lambda}_s$ [cm]	$\tau \cdot 10^{12}$ [s]
H_2O	70	0.88 ± 0.03	4.6
5	66	0.97 ± 0.08	5.1
10	62	0.98 ± 0.06	5.2
20	57	1.23 ± 0.06	6.5
30	52	1.37 ± 0.07	7.2
50	36	1.73 ± 0.17	9.1

was determined from the standard deviation of the slope. Table 1 contains the measured and calculated values. The 4th column of the Table contains the values of the time of relaxation calculated on the basis of

$$\tau = \frac{\bar{\lambda}_s}{6 \cdot \pi \cdot 10^{10}} \quad [\mathrm{s}] \tag{4}$$

The data show that with increasing concentration both the wavelength of relaxation of the water phase and the time of relaxation of the dipole rotation of water increase significantly. In a 50 per cent solution it is about twice as long as the value measured in water.

According to Table 2 the σ_{om} values determined on the basis of the point of intersection of the straight lines and the ordinate in Fig. 1 are larger than the σ_0 value of the sound frequency conductivity measured at 20 kHz. In our previous work (Masszi, Örkényi, 1967a) we already pointed out that sound-frequency

Table 2

Comparison of conductivity measured at 20 kHz (σ_0) and conductivity extrapolated from microwave data for "O" frequency (σ_{om}). (λ_s is the frequency of relaxation of the dispersion bringing about the increase of conductivity)

Conc. per cent	10^{-3}[ohm^{-1}cm^{-1}]		$\sigma_{om} - \sigma_0$	$\frac{\sigma_{om} - \sigma_0}{C}$	λ_s [cm]
	σ_0	σ_{om}			
H_2O	10^{-1}	0	—	—	—
5	2.7	4.5	1.8	0.36	139
10	4.5	9.2	4.7	0.47	106
20	6.3	12.8	6.5	0.32	158
30	6.0	14.5	8.5	0.28	179
50	2.5	11.5	9.0	0.18	278

conductivity arises from ionic conductivity corresponding to the 3 mg/g ash content of the gelatine and it can be significantly decreased by removing ash from gelatine. σ_{om} extrapolated on the basis of microwave data presumably arises from a process of relaxation occurring at lower frequency, as it is proved also by the direct measurements of Pennock and Schwan (1969) performed in haemoglobin solutions.

The difference between the conductivities measured at high- and low-frequencies, as compared with the frequency of relaxation, i.e. the ($\sigma = \sigma_{om} - \sigma_0$)

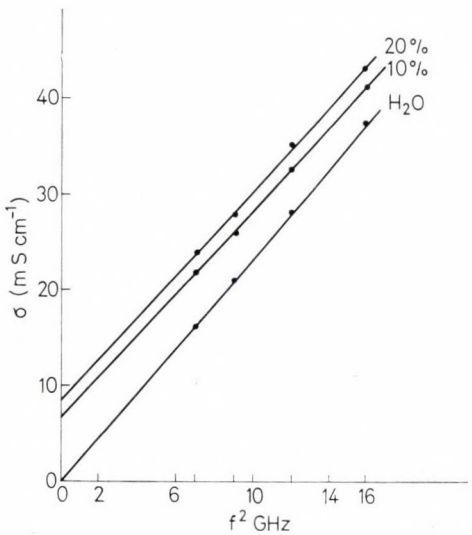

Fig. 2. Frequency-dependence of microwave conductivity of distilled water and of 20 to 30 per cent gelatine solutions at 20°C

value, is in connection with the decrease of dielectric constant in the range of relaxation ($\Delta\varepsilon'$) and with the wavelength of relaxation (λ_s) according to

$$\sigma = 10^3 \cdot \frac{\Delta\varepsilon'}{60\lambda_s} \text{ [m ohm}^{-1}\text{ cm}^{-1}\text{]}. \tag{5}$$

On the basis of the measurements of Schwan (1965), Grant (1965), Pennock and Schwan (1969), the $\Delta\varepsilon$ value corresponding to an $0.3 \cdot \frac{\text{g protein}}{100\text{ ml solution}}$ was used. The values of λ_s shown by the 6th column of Table 2 were estimated from equation (5). The relatively large standard deviation of the data can be explained by the approximating character of the calculations. However, our data unambiguously demonstrate that a further range of relaxation can be observed in gelatine solutions at a two orders of magnitude lower frequency.

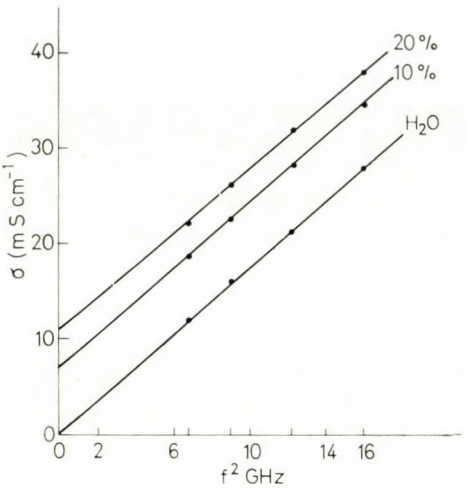

Fig. 3. Frequency-dependence of microwave conductivity of distilled water and of 20 to 30 per cent gelatine at 30°C

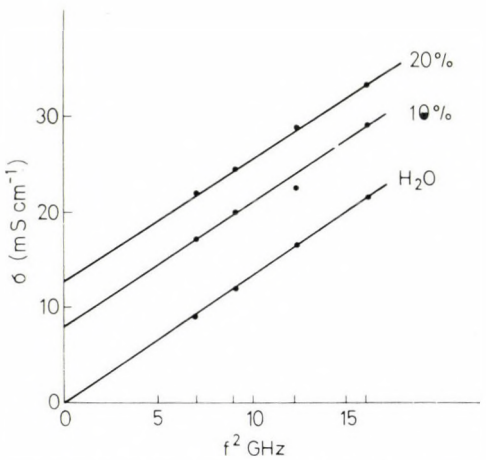

Fig. 4. Frequency-dependence of microwave conductivity of distilled water and of 20 to 30 per cent gelatine at 40°C

As the wavelength of relaxation of common water also shows marked temperature-dependence, we examined how the wavelength of relaxation would change as a function of the temperature in 10 and 20 per cent solutions. Figures 2, 3 and 4 show the data of measurements of solutions at 20, 30 and 40°C, respectively. The calculated values of the wavelength of relaxation are summarized in Table 3.

Table 3

Temperature-dependence of the wavelength of relaxation of 10 and 20 per cent gelatine solutions

°C	$\bar{\lambda}_s$ [cm]			$\bar{\lambda}_s/\bar{\lambda}_0$	
	H_2O	10 per cent	20 per cent	10 per cent	20 per cent
20	1.72	1.94	2.26	1.13	1.31
30	1.29	1.48	1.36	1.15	1.27
40	1.07	1.23	1.27	1.14	1.28
50	0.88	0.98	1.23	1.12	1.38
		Average:		1.14	1.31

Discussion

1. The gradual increase of the wavelength of relaxation and of the time of relaxation of the water phase shown by Table 2 indicates that the boundness of the water gradually increases with increasing gelatine concentration. According to the theory of reaction kinetics of Eyring, the energy of activation of dipole rotation, ΔE, and its entropy of activation ΔS have the following connection with the time of relaxation (Glasstone et al., 1941):

$$\frac{1}{\tau} = \frac{kT}{h} e^{\Delta S} \cdot e^{-\frac{\Delta E}{kT}} [S^{-1}], \tag{6}$$

where k is the Boltzmann constant,
h is the Planck constant
T is the absolute temperature.

The data of Table 3 show that the quotient of the time of relaxation of the water phase and the distilled water $\left(\frac{\lambda_s}{\lambda_{s0}} = \frac{\tau}{\tau_0}\right)$ does not depend significantly on the temperature. From this we can conclude, on the basis of equation (6) that the slowing down of the dipole rotation is not the consequence of the change of the ΔE value connected with the energy of binding of H-bridges, but the increase in the time of relaxation is brought about by the decrease of the ΔS activation entropy. The statement that with increasing gelatine concentration the average time of relaxation of dipole rotation increases and, contrasting with this, the entropy of activation decreases corresponds to the idea of the so-called "multi-layer" adsorption. According to this the boundness of the water adsorbed in several layers is expressed by the decrease of the entropy of rotation, and the boundness of water gradually decreases when the water is moving off the surface of the protein (Ling, 1965).

2. According to the data of Table 2, a further range of dispersion can be demonstrated also in gelatine solutions, similarly to other polyelectrolyte solutions. Its wavelength of relaxation is two orders of magnitude longer than that of free water. The process bringing about the dielectric relaxation has not been yet clarified. Pennock and Schwan (1969) concluded from their measurements performed in haemoglobin solutions that a dipole rotation of 0.15 to 0.25 g bound water/gHb could be observed in the dm range. Hoekstra and Doyle (1971) explain the manifestation of dispersion by the fluctuation of H-bridges connected to polyelectrolytes. The proton conduction on the surface described by Kirkwood and Shumaker (1952) cannot be excluded as an explanation of the process of relaxation.

On the basis of our investigations and of the data in the literature the dielectric characteristics of the water phase of polyelectrolyte solutions at the dm wavelength is determined by the direct interaction of protein and water. The microwave range can give information about the boundness of the whole water phase.

Appendix

1. In deducing equation (3) we started from the Debye (1929) equation, according to which:

$$\varepsilon'' = \frac{\Delta\varepsilon'}{1 + \left(\frac{\lambda_s}{\lambda}\right)^2} \cdot \frac{\lambda_s}{\lambda} + \frac{\sigma_{om}}{2\pi f \varepsilon_0}, \tag{7}$$

where

$$\varepsilon_0 = \frac{1}{4 \cdot \pi \cdot 9 \cdot 10^{11}} \left[\frac{A \cdot S}{V \cdot \mathrm{cm}}\right] \tag{8}$$

ε_0 is the dielectric constant of the vacuum. Considering that

$$\frac{1}{\lambda} = \frac{f}{3 \cdot 10^{10}} \ [\mathrm{cm}^{-1}] \tag{9}$$

$$\sigma = 2\pi f \varepsilon_0 \varepsilon'' \ [\mathrm{ohm}^{-1}\,\mathrm{cm}^{-1}] \tag{10}$$

and at the wavelength used by us:

$$\left(\frac{\lambda_s}{\lambda}\right)^2 \ll 1, \tag{11}$$

$$\sigma = \frac{\Delta\varepsilon' \cdot \lambda_s}{5.4 \cdot 10^{22}} f^2 + \sigma_{om} [\mathrm{ohm}^{-1}\ \mathrm{cm}^{-1}]. \tag{12}$$

If the frequency is measured in GHz and the conductivity in mScm^{-1} taking (10^{-3} $\mathrm{ohm}^{-1}\,\mathrm{cm}^{-1}$) as a unit:

$$\sigma = \frac{\Delta\varepsilon'\lambda_s}{54} \cdot f^2 + \sigma_{om} \text{ [mScm}^{-1}\text{]}. \tag{13}$$

2. In polyelectrolyte solutions, after resolving the water phase to n layer in which the characteristics of the different layers are $\Delta\varepsilon_i$ and λ_{si}, equation (13) can be written in the following form:

$$\sigma = \frac{\Delta\varepsilon'}{54} \sum_{i=1}^{h} \frac{\Delta\varepsilon_i'}{\Delta\varepsilon'} \cdot \lambda_{si} + \sigma_{om} \tag{14}$$

Considering the former data, according to which, as first approximation, the dielectric constant of the water phase agrees with the dielectric constant of the free water (Pennock, Schwan, 1969; Masszi, 1970),

$$\frac{\Delta\varepsilon_i'}{\Delta\varepsilon'} = \frac{n_i}{n}. \tag{15}$$

where n_i means the number of water molecules in the "i" layer, and n is the number of all water molecules. From the comparison of equations (12) and (13) as well as (14)

$$\bar{\lambda}_s = \frac{\sum_{i=1}^{h} n_1 \cdot \lambda_{si}}{n} \tag{16}$$

i.e. the wavelength of relaxation determined by us corresponds to the mathematical average of wavelengths of relaxation weighted with the number of molecules in the different layers.

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Application a Three Compartment Tracerkinetic Model for Comparing the K^+, Rb^+ and Cs^+ Transport of Erythrocytes*

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The ion transport of rat erythrocytes was investigated by comparing the characteristic parameters of K^+, Rb^+ and Cs^+ transport. ^{42}K, ^{86}Rb and ^{137}Cs isotopes were added to freshly drawn blood at 37°C temperature, and then the time dependence of the activity both of the plasma and of the erythrocytes was measured. The results of measurements were evaluated using a three-compartment linear tracerkinetic model. An analog computer was used for solving the differential equation system stated on the basis of the model. The four transport coefficients quantitatively characterizing the plasma ↔ membrane and membrane ↔ intercellular space transitions were calculated from the parameters of theoretical curves perfectly fitting to the experimental points in the case of all three ions, and the differences between them were interpreted.

Introduction

In the biophysical sense a membrane is a molecular system which by its structure and internal energy source maintains and regulates by molecular interactions the substance and energy transport across it.

On the above basis the aim of biophysical researches on membranes is first of all to discover the *molecular structure* with different methods (electron microscope, X-ray diffraction, infrared spectroscopy, ESR, NMR) on the one hand, and studying the *transport kinetics* to get informations about the mechanism of membrane function with the aid of kinetic models (multicompartment, phenomenologic-, molecular model). Direct examinations are still handicapped nowadays by several technical difficulties therefore in spite of the rapid development achieved in the recent years (Chapman, 1970) relatively little is known about the structure of membranes. At the same time the application of radioactive isotopes and especially the possibility of evaluation by means of computers make easier the kinetic analysis of processes involved in the composite system comprising the membrane and the two media separated by this latter.

The comparison of the characteristic parameters of K^+, Rb^+ and Cs^+ transport offers a very good opportunity for the study of the mechanism of ion transport taking place in biological membranes.

* Based on a lecture read at the 6th Congress of the Hungarian Biophysical Society.

Under physiological circumstances the concentration of Rb^+ and Cs^+ ions as well as their biological role can be neglected when compared with that of K^+ and Na^+. Their behaviour therefore is only slightly elucidated. On the other hand it is known that in biological systems Rb^+ behaves similarly to K^+, a fact utilized in several radioisotope tests (Love, Burch, 1953; Kahn, 1962; Hashimoto, Yoshikawa, 1963; Bakkeren, Bonting, 1968). From the point of view of biological processes Cs^+ ion is of minor importance, but it is one of the most important objects of radiation protection investigations in connection with the peaceful use of atomic energy as well as in nuclear weapon tests (Yamagata, Iwashima, 1966;, Yamagata, et al., 1966; Stather, 1970).

In our experiments the transport of K^+, Rb^+ and Cs^+ ions – having similar physical characteristics – was investigated in red blood cells of rats by means of ^{42}K, ^{86}Rb and ^{137}Cs isotopes, respectively.

Methods

The freshly drawn, heparinized blood of albino rats kept on normal diet and weighing about 200 g was used in the experiments. The blood collected was placed in a thermostate warmed up to 37°C, and the radioactive isotope was added to give a final concentration of 0.2 to 0.4 μCi/ml blood. The ^{137}Cs applied here was carrier free and the carrier of ^{42}K and ^{86}Rb meant a maximum extracellular concentration of 0.1 meq/l. After incubation at cautious stirring, 2×1 ml samples were withdrawn at given intervals centrifugated and then the activity of the plasma and the red blood cells – which latters were washed three times with cold physiological salt – has been measured with a well-type scintillation counter.

Double-labelled samples were used in most of the experiments. The activity of the two isotopes was determined on the basis of their different half-life time.

The activity of the erythrocytes as well as that of the plasma were expressed in the per cent of the activity of the whole blood, taking the haematocrit into account. The haematocrit value was determined by centrifuging the blood in normal haematocrit tubes with about 4000 g for 15 min, taking the about 4 per cent trapped plasma into account.

Under the experimental conditions outlined above, the blood can be considered as a closed system in which a steady state exists between plasma and erythrocytes. This state of dynamic equilibrium did not change even when radioactive isotopes were added, because ^{137}Cs was carrier-free and the carrier of ^{42}K increased the concentration of extracellular K^+ by 2 per cent only. In the case of ^{86}Rb the inactive Rb^+ added though changed the "Rb^+ steady state" but, according to our experiments, in concentration range of a 0.01 to 0.3 meq/l extracellular Rb^+, the percentage ^{86}Rb uptake of the erythrocytes was practically equal. From this fact we concluded that in physiological circumstances, the speed of Rb^+ transport is not determined by the Rb^+ concentration distribution, but by the potassium being present in an about 50 times higher concentration. By the way

this fact is in accordance with the conception that in the case of erythrocytes the three ions are transported by a common system. At the same time, the rate constants of Rb^+ and Cs^+ administered into the K^+ flux in tracer quantity are determined by the interaction between the different isotope ions and of the membrane, i.e. the results measured experimentally really reflect the differences between the transport of the three ions in the case of physiological steady state.

It must be also noted that in the case of simultaneous application of two isotopes – because of the very small concentration of radioactive isotopes – competitive inhibition could not be experienced.

According to the theory of isotope kinetics, it is possible to determine the kinetic constants characteristic of this state by measuring the decrease of the activity of the radioactive isotope added to the plasma.

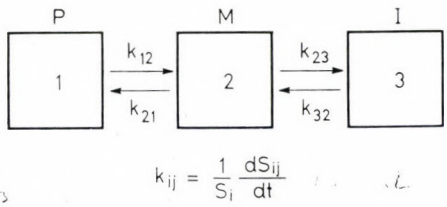

Fig. 1. Scheme of the three-compartment model. *P*: plasma, *M*: membrane, *I*: intracellular space, k_{12}, k_{21}, k_{23}, k_{32} are coefficients characterizing the transport between the different compartments

In order to evaluate the experimental data, the linear tracer kinetic models applied to similar investigation were used (Sheppard, Beyl, 1951; Sheppard, 1962; Defares, Sneddon, 1964). By means of the two-compartment model the behaviour of the three ions could be studied independently (Györgyi, Kanyár, 1971).

But when we evaluated our last measurements which were more exact and comprised a longer period of time, the fitting of the measured values to those calculated with the aid of the model of two compartments was not satisfactory. This we think to be caused by the fact that the membrane of the erythrocyte is separated from the intracellular space both structurally and functionally. Therefore – as a further simplest case – we assumed that, beside the plasma (*P*) and intracellular space (*I*), also the membrane represents a separate phase, a compartment (*M*), in the system. The model of three compartments obtained in this way offers an opportunity for a more detailed analysis of kinetic characteristics. The model applied can be seen in Fig. 1.

Considering our system of three compartments also as a closed one we described the change of the concentration of a certain ion in the different compartments with linear equations:

$$\frac{dS_1}{dt} = k_{21}S_2 - k_{12}S_1$$

$$\frac{dS_2}{dt} = k_{12}S_1 + k_{32}S_3 - k_{21}S_2 - k_{23}S_2$$

$$\frac{dS_3}{dt} = k_{23}S_2 - k_{32}S_3$$

S_i ($i = 1, 2, 3$) is the concentration of a given ion in the compartment i, k_{12}, k_{21} . . . in general with the notation k_{ij} characterize the ion transport between the different compartments. The latter values are constant under the experimental conditions applied here.

The differential equations can be written not only for the inactive substance, but also for the corresponding radioactive isotopes (Sheppard, 1962). In the present case the plasma was considered as the 1st compartment to which the radioactive isotope was then added, the quantity of which was expressed in percentage. So we could use $S_1(0) = 100$, $S_2(0) = S_3(0) = 0$ as an initial condition for solving the system of equations.

After solving the system of differential equations the expression

$$S_i = A_0 + Ae^{-\alpha t} + Be^{-\beta t}$$

was obtained for the change of the concentration in compartment i, where A_0, A, B, α, and β constants are the algebraic functions of the k_{ij} transport coefficients and of the initial conditions. By approximating the experimentally measurable $S_i = S_i(t)$ curve with such a function, the coefficients and exponents of the exponentials and then the k_{ij} coefficients characterizing the ion transport can be calculated. When solving of the differential equations and curve-fitting was done with a MEDA-41 analog computer, the transport coefficients could be read directly from the positions of the potentiometers.

Experiments

The results of an experiment with ^{42}K obtained with the aid of an analog computer can be seen in Fig. 2.

The Figure shows the experimental values as well as the theoretical curves. The theoretical curves were fitted to the experimental points obtained for the activity of both the plasma and the membrane + intracellular space.

The change in activity of both the membrane (M) and the intracellular space (I) was outlined separately, which cannot be measured experimentally.

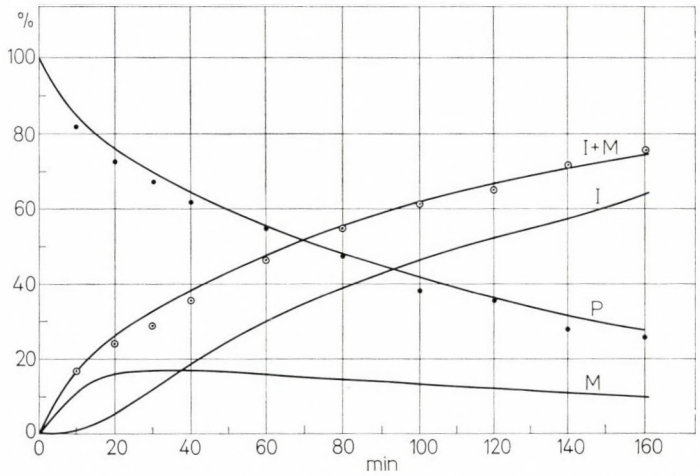

Fig. 2. Experimentally measured ^{42}K activity values of erythrocyte (⊙) and plasma (•) and theoretical curves obtained by means of an analog computer. (*P*: plasma, *M*: membrane, *I*: intracellular space, $I + M$: curve describing the time dependence of erythrocyte activity.)

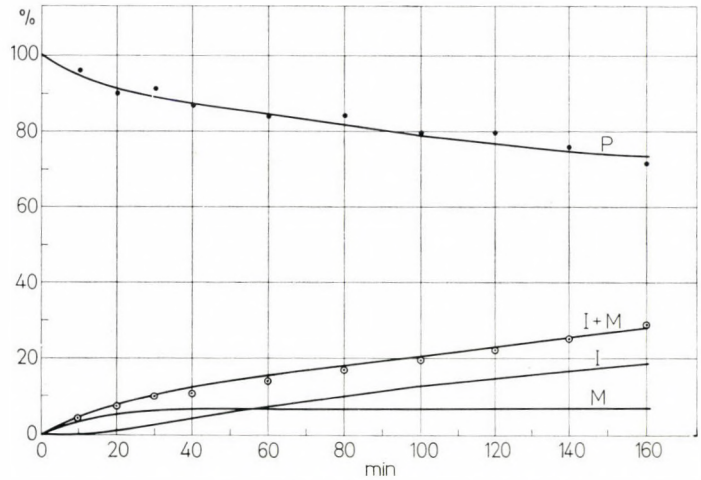

Fig. 3. Experimentally measured ^{137}Cs activity values of erythrocyte (⊙) and plasma (•) and theoretical curves obtained by means of analog computer. (*P*: plasma, *M*: membrane, *I*: intracellular space, $I + M$: curve describing the time dependence of erythrocyte activity.)

For ^{86}Rb, curves very similar to the previous ones were obtained, while the change of activity of ^{137}Cs is shown in Fig. 3.

Table 1 summarizes the values of transport coefficients obtained by the analogous evaluation.

Table 1

	k_{12}	k_{21}	k_{23}	k_{32}
	$\times 10^{-4}$ sec^{-1}			
K^+	9.2	37	23	0.33
Rb^+	14	42	27	0.38
Cs^+	4.6	46	9.1	0.30

Discussion

When we compare the different ions, we find essential differences first of all in the case of k_{12} and k_{23}. If we assume that k_{12}, the coefficient of plasma-membrane transfer characterizes the extracellular ion-membrane interaction quantitatively, it can be said that Rb^+ ions have higher affinity to the outer surface of the membrane than have K^+ or Cs^+. This is corroborated also by our saturation kinetic examinations. At the same time the difference experienced in the case of k_{23} indicates that the dissociation probability of Cs^+ in the inner side of the membrane is much smaller than that of the other two ions. The differences can be made more expressive if we compare the transport coefficients to K^+.

	k_{12}	k_{21}	k_{23}	k_{32}
K^+	1	1	1	1
Rb^+	1.52	1.14	1.17	1.15
Cs^+	0.50	1.24	0.40	0.91

If we compare Rb^+ and K^+, beside the already mentioned difference in k_{12}, the quasi-equal increase (15 per cent on the average) of the other three ones is characteristic, which unambiguously shows that the rate of the transport of Rb^+ is higher than that of K^+. The known behaviour of Cs^+ is shown in the present case by the difference in k_{12}. The lower values of k_{23} and k_{32} can be considered as further data, also characterizing the behaviour of Cs^+ different from that of the other two ions.

With the present accuracy of the experimental results the linear three-compartment model is satisfactory for their evaluation. Introduction of further independent parameters (e.g. through separating the membrane into two compartments) would result in a great increase of their uncertainty and so the evaluation would become less accurate.

However, the comparison of the four transfer coefficients of the examined three ions has made possible to compare also the transport mechanism of them; our aim is to throw light on the molecular processes and to interpret the differences on the level of molecular interactions. In our further work the results obtained with kinetic method will be used for solving the molecular model.

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Energy Consumption of Active Sodium Transport in Isolated Frog Skin

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Oxygen consumption of active sodium transport in the frog skin was investigated at various temperatures, pH values, and osmotic concentrations. By altering the parameters without irreversible damage of the tissue the authors found the oxygen consumption per unit sodium transport to be the lowest at physiological values.

The results were analyzed thermodynamically. Minimum energy dissipation in the respective stationary states was achieved at different absolute levels. The efficiency of energy conversions exhibited the highest value under physiological conditions.

Introduction

It is a general property of living organisms that a high energetical efficiency of their biological processes is limited to a narrow range of the parameters of their environment. The experiments to be reported were designed to obtain a relatively precise description of this biological principle by the quantitative analysis of the optimum range in an isolated biological system. A biological object was selected in which an active process could be well measured and also the quantity of energy consumed for this process could be determined. From the numerous possibilities we chose the active sodium transport in frog skin. With the method of Ussing and Zerahn (1951) the rate of sodium transport can easily be determined without affecting the functions of the skin. Precise information can be obtained on the energy portion used for transport by comparing the oxygen consumption of transporting and non-transporting skin pieces (Zerahn, 1956). In the experiments to be reported the oxygen consumption required for the active sodium transport in the frog skin was investigated as a function of temperature, pH, and osmotic concentration.

Materials and methods

Isolated abdominal skin of frogs *(Rana esculenta)* weighing 50 to 70 gr was used for the experiments. In order to attain substrate-depletion and to eliminate endogenous hormone effects the skin was kept in 0.6 per cent solution of

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NaCl for 3 hours before the experiment. With the aid of a pattern the skin was cut into 3 parts: 1 piece of the size of 3 cm^2 served for the estimation of sodium transport rate and 2 pieces, 1.5 cm^2 each, were used to determine oxygen consumption.

The rate of active sodium transport was estimated by the short-circuit technique of Ussing and Zerahn (1951). Chambers for estimating the sodium transport were continuously oxygenized. Temperature was stabilized by means of an external water bath. The incubation periods lasted one hour.

Oxygen consumption was determined by Warburg's direct manometric method (Umbreit et al., 1957). The amount of energy required for sodium transport was calculated on the basis of oxygen consumption determined in transporting and non-transporting skins. Blocking of transport was carried out by means of Amiloride (10^{-5} M) (SK 870, Merck, Sharp et Dohme). The dose applied proved sufficient to reduce the membrane potential almost to zero, while the μA values were inmeasurably low. Each vessel contained 80–100 mg of tissue (wet weight). Oxygen consumption was determined at the end of the one-hour incubation periods.

Ringer's solution of the following composition was used: NaCl 72 mM; KCl 1.9 mM; $CaCl_2$ 0.9 mM; Na_2HPO_4 16 mM; NaH_2PO_4 4 mM (pH 7.4; osmotic concentration 206.5 milliosmol/litre; sodium concentration 108 mEq/litre). The solution also contained 10^{-3} M pyruvate.

The results are related to values of dry weight.

I. Change in the temperature:

The rate of sodium transport and the energy consumption belonging to it were determined at 15, 20, 25, 30, 35, 40, and 45°C. Six experiments were carried out at each temperature. For technical reasons the experiments at any given temperature were performed at the same time (on the same day), but the experimental series were undertaken in the following turn: 25°C, 35°C, 45°C, 15°C, 20°C, 30°C, 40°C. The experiments were performed within a month (in May, 1971).

The effect of temperature on the rate of sodium transport was investigated also at continuously increasing temperature. In these experiments the skin was cut into 2 parts and while one piece was kept at 25°C the other one was gradually heated from 15 to 45°C. The temperature of the incubation media increased with an average of 5°C in 10 minutes.

II. Change in the pH:

Six experiments were carried out in incubation media of pH 6.8, 7.4, and 8.0, respectively. Changing the pH was carried out by altering the ratio of the constituents of phosphate buffer. The Ringer solution was kept isosmotic in each case, temperature was 25 °C.

III. Change in the sodium and osmotic concentration:

The Na^+ (osmotic) concentration of the incubation media was adjusted by increasing and decreasing the quantity of NaCl. 6–6 experiments were carried out in which the Ringer solution had a Na^+ concentration of 72 mEq/l or 144 mEq/l. The corresponding changes in the osmotic concentration will also

be indicated. The incubating media were kept at pH 7.4 and on 25 °C.

The experiments dealing with the effect of changes in pH and Na^+ (osmotic) concentration were carried out within 16 days (in July, 1971). The individual experiments followed one another in a random turn. Just as in the case of experiments with temperature alteration, the effect of continuous change in the pH and Na^+ (osmotic) concentration on active sodium transport rate was investigated here, too.

Oxygen consumption per unit sodium transport was calculated separately for every single skin in order to eliminate the effect of individual variations.

Statistical analysis of the results was carried out by using Student's paired-simple *t*-test.

Results

I. Effect of changes in temperature on the active sodium transport and oxygen consumption of the frog skin (Table 1).

Table 1

Effect of changes in temperature on the active sodium transport and oxygen consumption of the frog skin

Temperature °C	*n*	Mean μA/3 cm^2	Q_{O_2} Ringer* μl/mg/hr	Q_{O_2} Amiloride* μl/mg/hr	Transport Q_{O_2}* μl/mg/hr
15	6	17	0.47±0.12	0.22±0.13	0.25±0.13
20	6	35	0.74±0.15	0.55±0.16	0.20±0.03
25	6	70	0.80±0.26	0.61±0.23	0.18±0.07
30	6	77	1.05±0.22	0.88±0.21	0.16±0.11
35	6	62	1.08±0.12	0.82±0.19	0.26±0.17
40	6	14	1.03±0.16	0.83±0.14	0.20±0.07
45	6	9	1.18±0.25	0.90±0.28	0.27±0.19

* Mean ± S.D.

1. By varying the temperature continuously the rate of the active sodium transport exhibited marked temperature dependence. The highest μA values were obtained at 25°C and 30°C, while the lowest ones at 15°C and 45°C (Fig. 1). A similar pattern was observed in experiments measuring the sodium transport rate and the oxygen consumption simultaneously.

Sodium transport measured simultaneously with tissue respiration displayed a constant rate throughout the one-hour experimental period. The decrease in the transport caused by higher or lower temperatures appeared very quickly (within a few minutes) and was completely reversible, irrespective of the way of temperature changes. The importance of these observations will be discussed later.

2. In the temperature range from 15 to 45°C the rate of oxygen consumption continuously increased both in the Ringer and the Amiloride-Ringer solutions. The coefficient of correlation between oxygen consumption and temperature was 0.739 ($p < 0.001$) in the Ringer solution and 0.565 ($p < 0.001$) in the Amiloride-Ringer.

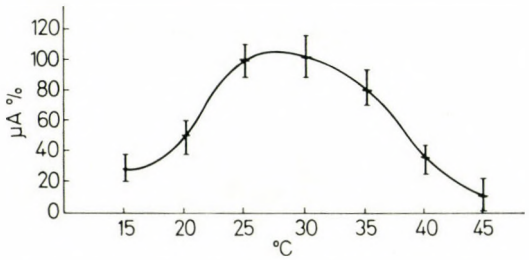

Fig. 1. Alteration of the active Na^+ transport by continuous increasing of temperature. (Data are related to the transport rate measured at 25°C.) (Mean ± S. D.)

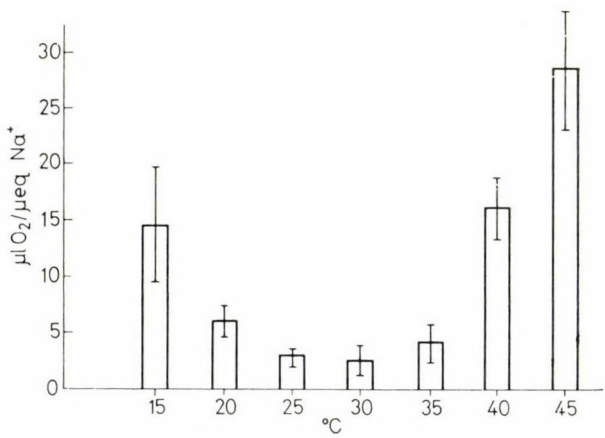

Fig. 2. Oxygen consumption per unit Na^+ transport between 15°C and 45°C. (Mean ± S. D.)

3. Oxygen consumption required for total sodium transport (Q_{O_2} Ringer − Q_{O_2} Amiloride-Ringer) significantly differed from zero in the whole range of temperature. Changes of temperature failed to affect this value, though a tendency of increase was observed at extreme temperature values.

4. Oxygen consumption per unit Na^+ transport, as shown in Fig. 2, was about five times higher at 15°C than at 25°C, whereas at 45°C it exceeded ten times the value measured at 25°C. The changes were statistically significant. No significant difference was found between the values observed at 25, 30, and 35°C, respectively.

5. Sodium transport and the oxygen consumption attached to it displayed a linear relationship at each temperature. The data obtained at 25 and 45°C represent an example for this (Fig. 3). (The temperature dependence of the oxygen

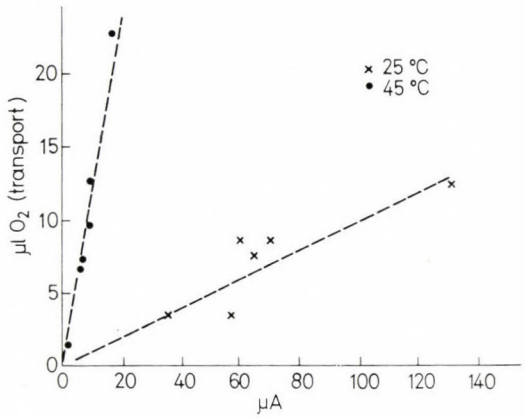

Fig. 3. Relationship between the active sodium transport and the oxygen consumption required for it at 25°C and 45°C

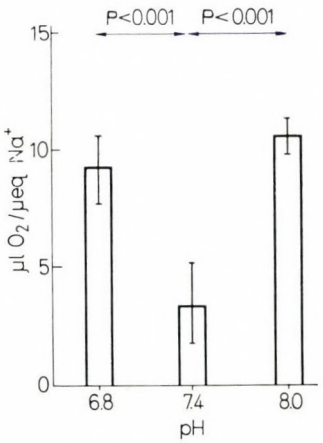

Fig. 4. Oxygen consumption per unit Na^+ transport at pH 6.8, 7.4, and 8.0 (Mean ± S. D.)

consumption per unit sodium transport is well represented also in this figure by the slopes of the lines.)

II and III. The effect of changes in pH and in Na^+ (osmotic) concentration on the active sodium transport and oxygen consumption of the frog skin (Table 2 and Table 3).

Table 2

The effect of changes in pH on the active sodium transport and oxygen consumption of the frog skin

pH	*n*	Mean $\mu A/3\ cm^2$	Q_{O_2} Ringer* $\mu l/mg/hr$	Q_{O_2} Amiloride* $\mu l/mg/hr$	Transport Q_{O_2}* $\mu l/mg/hr$
6.8	6	25	0.71±0.26	0.48±0.14	0.23±0.16
7.4	6	50	0.74±0.20	0.59±0.19	0.16±0.09
8.0	6	23	0.63±0.11	0.41±0.12	0.21±0.05

* Mean ± S.D.

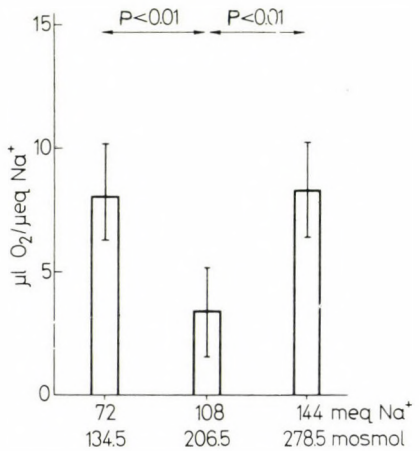

Fig. 5. Oxygen consumption per unit Na^+ transport at Na^+ concentrations of 72 mEq/1, 108 mEq/1, and 144 mEq/1 (Osmotic concentrations of 134.5, 206.5, and 278.5 milliosmol/1.) (Mean ± S. D.)

Table 3

The effect of changes in Na^+ (osmotic) concentration on the active sodium transport and oxygen consumption of the frog skin

Na^+ conc. Osmotic conc.	*n*	Mean $\mu A/3\ cm^2$	Q_{O_2} Ringer* $\mu l/mg/hr$	Q_{O_2} Amiloride* $\mu l/mg/hr$	Transport Q_{O_2}* $\mu l/mg/hr$
72 mEq/l Na^+ 134,5 mosm/l	6	20	0.46±0.10	0.32±0.09	0.14±0.04
108 mEq/l Na^+ 206,5 mosm/l	6	50	0.74±0.20	0.59±0.19	0.16±0.09
144 mEq/l Na^+ 278,5 mosm/l	6	22	0.48±0.08	0.32±0.06	0.16±0.05

* Mean ± S.D.

When examining the effect of pH and Na^+ (osmotic) concentration on energy consumption, a pH and concentration range was applied within which the transport rate was kept constant throughout the one hour incubation period, and any decrease was reversible.

The dependence of oxygen consumption per unit sodium transport on pH and Na^+ (osmotic) concentration is shown in Fig. 4 and Fig. 5.

Discussion. Theoretical conclusions

I. Comparison of the present experimental results with the data in the literature:

1. Amiloride was used for blocking active sodium transport. In accordance with the data obtained by others (Crabbé, Ehrlich, 1968; Ehrlich, Crabbé, 1968). the applied concentration reduced also in our experiments the membrane potential almost to zero. Amiloride, a specific and reversible inhibitor of active sodium transport in the frog skin exerts its effect by hindering the passive influx of sodium ions into the transporting cells (Ehrlich, Crabbé, 1968). According to Parisi and Bentley (1970) Amiloride has no specific metabolic effect different from the Na^+-deficient milieu.

2. The dependence on temperature, pH, and osmotic conditions of the active Na^+ transport in the frog skin was observed by several authors (Dalton, Snart, 1967; Snell, Leeman, 1957; Ussing, 1965; Davson, 1959, etc.). The magnitude of changes and the site of optimum values were, of course, not identical since the experiments were carried out on diverse species and in various seasons.

3. The values we obtained for the total oxygen consumption of the frog skin agree well with the data obtained by other authors using different methods (Zerahn, 1956; Zerahn, 1961; Leaf, Renshaw, 1957).

4. Investigating the ratio of the active Na^+ transport to the oxygen consumption attached to it, Zerahn (1956, 1961) found that the transport of 10 moles of sodium ions required 1 mole of oxygen. According to the experiments of Leaf and Renshaw (1957) the transport of every 2 to 13 moles of sodium requires 1 mole of oxygen. (The temperature was about 25°C.) In our own experiments the ratio $O_2 : Na^+$ was 1 : 8 at 25°C. Zerahn (1961) investigated the oxygen requirement of the sodium transport using a great variety of experimental conditions, but failed to observe any significant difference.

5. In the theoretical discussion oxygen consumption is regarded as a quantity proportional to energy consumption. This assumption is based on the following arguments:

a) According to the literature (Zerahn, 1956) the anaerobic metabolism of the frog skin is negligible in incubation media saturated with oxygen.

b) CO_2 production of the skin was determined in several experiments both under normal and unfavourable conditions. RQ values of 1.2 were consistently obtained indicating that the $Q_{O_2} : Q_{CO_2}$ ratio was constant, and that the substrate depleted skin tissue utilized pyruvate.

II. Theoretical conclusions

The system discussed can be characterized according to the general theory of transport processes (Gyarmati, 1970) by the following quantities:

j_{Na} – the current density of the transported Na^+ ions (moles/cm^2 · sec)
x^*_{Na} – grad $\tilde{\mu}_{Na}$ – the thermodynamical force conjugated to j_{Na} in energy picture (cal/mole · cm) ($\tilde{\mu}_{Na}$ = the electrochemical potential of sodium ions)
Ψ_{Na} – the share of the active transport of Na^+ ions in the specific energy dissipation (cal/cm^3 · sec)
p, T, pH, c_{Na} ... – fixed external parameters as boundary conditions.

We have the following informations on these quantities:

j_{Na} – is equal to the experimentally measured Na_+ transport rate per unit surface area
x^*_{Na} – can approximately be calculated from the height of the concentration barrier, the thickness of the membranes, and the membrane potential
Ψ_{Na} – is proportional to the share of the Na^+ transport in the oxygen consumption, providing that energy dissipation : oxygen consumption and volume : dry weight ratios are constant.

The environmental parameters are regarded as given ones in each single experiment.

In course of the experiments it could be ascertained that under fixed environmental parameters the rate of active Na^+ transport proved to be constant during the period of the measurement (Fig. 6). Thus the transporting system can be considered stationary during the periods in question. Stationary systems can be characterized thermodynamically with the minimum of energy dissipation. This is a fundamental physical fact and, as such, it is naturally valid also for biological systems. However, attention should be payed to the fact that varying the external

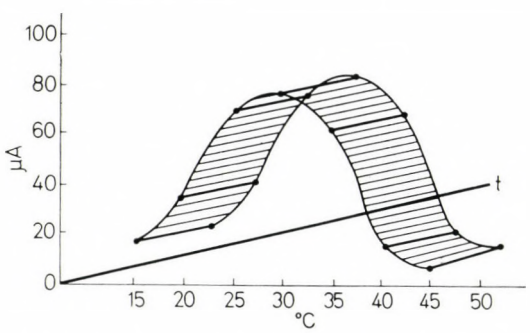

Fig. 6. Sodium transport rate at different temperatures during the 1 hour measurement periods

parameters the transporting system reaches newer and newer stationary states corresponding to the new boundary conditions, all of them – even those under highly unfavourable conditions – meaning a minimum of energy dissipation with respect to the instationary processes leading to the given stationary state. (The environmental parameters were varied just in such a way that no irreversible change occurred in the structure of the skin and the active sodium transport could be stabilized – even if at different absolute levels.)

It is advantageous to define a new quantity:

$$\xi_{Na} = \frac{\partial \Psi_{Na}}{\partial j_{Na}} \tag{1}$$

which can be called – by analogy with the thermodynamical force – the force biologically conjugated to j_{Na}.

Accepting the theoretically obvious linear relationship between the transport rate and the oxygen consumption required for it (Fig. 3), (1) simplifies into

$$\xi_{Na} = \frac{\Psi_{Na}}{j_{Na}} . \tag{2}$$

Thus, taking the volume : surface area : dry weight ratios into account – the force biologically conjugated to j_{Na} can be considered proportional to the oxygen consumption per unit Na^+ transport shown in Figures 2, 4, 5 as a function of the various environmental parameters.

From (2) it can be obtained:

$$\frac{x^*_{Na}}{\xi_{Na}} = \frac{j_{Na} \cdot x^*_{Na}}{\Psi_{Na}} . \tag{3}$$

The numerator of the right hand side is equal to the theoretical energy dissipation belonging to the transport, whereas the denominator means the measurable quantity of energy actually dissipated by the "driving" fluxes (in our case presumably the various chemical reactions of metabolism). Thus the quotient is just the efficiency of energy conversion defined by Kedem and Caplan (1965), that is to say,

$$\eta_{Na} = \frac{x^*_{Na}}{\xi_{Na}} . \tag{4}$$

Based on this formula the efficiency of the Na^+ transport could be calculated if x^*_{Na} were known. Unfortunately the facilities are very limited in this respect, since the concentrations and activity coefficients in the transporting cells, moreover, the exact geometry of the membranes are very poorly known. Hence the most careful calculations mean no more than rough approximations.

Another characteristic quantity of active transport more closely related to ξ_{Na} is defined by Essig and Caplan (1968). They define the efficacy of flow for a given cationic species as

$$\varepsilon_{j+} = \frac{j_+}{j_r A} , \tag{5}$$

where $j_r A$ is equal to the energy dissipation of the coupled chemical reactions. Obviously this quantity is just the reciprocal of ξ_{Na}. Nevertheless we have to mention that that is originally defined for the theoretical characterization of a two-flow system for the case of $x_+^* = 0$, while ξ_{Na} is used by us as an experimentally measurable quantity characterizing a complex biological system in general.

As a summary of our experimental results it may be concluded that there exists a range in the field of the environmental parameters where the force biologically conjugated to the transport is minimum, or in equivalent terms, the efficiency of energy conversion (or the efficacy of flow) is maximum. Moving away off this range, ξ_{Na} increases, η_{Na} (or ε_{Na}) decreases.

The biochemical explanation of the observed phenomenon requires further investigations.

Acknowledgement

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Present and Prospects of Biophysics

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1. Biophysics is lagging behind biochemistry in scientific common knowledge. 2. Today's biophysics can overtake biochemistry by its results produced in fields as e. g. a) structure, b) theoretical biophysics, c) biocybernetics. 3. Biophysics is superior to biochemistry, on the one hand, and will in future fuse with biochemistry, on the other, in many questions dealt with in chapter 2 and also in problems as e. g. a) bound water, bound K, Na, Ca etc. b) "Water in Biology", c) biofrequencies, d) nuclear biology.

Beginning this "Study" I should like to emphasize that it intends much less to establish an experimental fact or to lay down some rules than to call attention to certain problems in the general state of our science.

1. To do so let us first of all compare the development of biophysics with that of biochemistry.

1.1. Both began their courses contemporaneously in the 16th and 17th centuries; furthermore the Ergebnisse der Physiologie, this very famous Annual Review, was published in two parts yearly in 1902–1904: one volume for biochemistry, and one for biophysics. Since then biophysics has fallen in the background, and today it seems that biophysics is lagging behind biochemistry in scientific importance.

Three examples: in 1971 professor Petrovski [1], Minister of Health in the Soviet Union gave the following picture of the future tasks of Soviet medical science: "We will support and widen those scientific institutions which occupy themselves with working out the basic theoretical problems of medical science: the physiological, pathophysiological, biochemical, morphological, immunological, pharmacological and genetical institutes." Biophysics was not mentioned.

Another example refers to the meeting of the British Biophysical Society (1969), where Bernard Katz [2], Professor of Biophysics at the University College, London, discussing the unfavourable situation of biophysics, emphasized the need to "define" biophysics. That happened about fifteen years after Katz himself had published his good review entitled "Biophysical Aspects of Neuro-Muscular Transmission" [3]. This indecision in estimation of our science shows the real situation of biophysics in the family of natural sciences.

* The invited lecture delivered by me at the IVth International Biophysical Congress in Moscow (7–14 August, 1972) was based on this "Study".

The third example: "Current Contents" [4] reports upon "Life Sciences featuring Animal et Plant Science, Behavioural Science, Biochemistry, Biology et Zoology, Botany et Entomology, Chemistry et Polymer Science, Clinical Medicine, Cytology et Pathology, Dental et Bone Research, Dermatology, Drug Research, Ecology, Endocrinology, Experimental Medicine, Genetics et Pediatrics, Geriatrics et Gerontology, Haematology, Medicinal Chemistry, Microbiology et Virology, Molecular Biology, Nutrition, Organic Chemistry, Pharmaceutical Chemistry, Pharmacology, Physiology, Radiation Science, Surgery." Biophysics is missing.

1.2. To tell the truth biophysics has been overshadowed by biochemistry having produced important *scientific discoveries*, e.g. the biopolymers, vitamins and hormones. An interesting example of the latter was demonstrated at the XVIth International Physiological Congress in 1938, when a vial was shown containing "maternal love" as the attendents were informed. Biophysics has not produced such very impressive discoveries.

1.3. When biochemistry already had *university chairs* all over the world, biophysicists began to form their international organization in 1961 in Stockholm, where I very strongly proposed that our new organization should occupy itself with the education of biophysics. For, the future of this science depends on how fast and in what number young biophysicists will be educated. Now, there are 6 university chairs for biophysics in my country, Hungary, inhabited by 10 millions. What is the situation in other countries?

1.4. The set-back of biophysics in comparison with biochemistry appears also from the *periodicals* dealing with the respective sciences. As an argument for this statement the well known Biochimica et Biophysica Acta could be mentioned: volume 1 of this Acta published biochemical and biophysical papers in the ratio of 7 : 3 in 1957; today this ratio could be estimated to be at least 10 : 1; but it is also true that the discrimination between biochemical and biophysical fields has lost its meaning for many papers.

On the other hand, biophysics has the Quarterly Review of Biophysics; this periodical, however, cannot counteract the unfavourable fact that papers of biophysical content are published in very different periodicals, thus preventing proper and fast information of biophysicists.

2. The present state

Comparing today's state and results of biophysics with those of biochemistry one can establish important advantages by which biophysics can overtake biochemistry.

2.1. First of all *structure* should be mentioned. As an explanation, events half a century ago should be recalled when the so-called lactic acid theory of muscle contraction was awarded the Nobel prize. The contraction was explained according to this theory by the biochemical fact of lactic acid production on the one hand, and by thermodynamics, i.e. by heat production, on the other. At that time I got permission to do research work in a scientific institute because I had

succeeded in explaining to its leader that both groundstones of this theory were wrong in principle. For the chief problem is the mechanism of contraction, and neither biochemistry nor thermodynamics is directly inter-linked with the mechanism of a process; in the same way as e.g. the chemistry and thermodynamics of autocars, aircrafts, motorboats and submarines are equal, the different mechanisms of their working depend on their different structures.

The importance of structure seems to be generally accepted in biophysical research today, and that appears e.g. from the newer hypotheses of muscle contraction. Notwithstanding, all these trials are one-sided, neglecting fundamental long-known experimental facts, as e.g. shortening of the anisotropic bands during contraction, failing contractility at normal action currents due to hypertonicity, tetanic contraction without normal spikes due to hypotonicity, etc. etc.

2.2. Another general field of biology in which biophysics is superior to biochemistry is the so-called *theoretical biology or theoretical biophysics*. In this field, suffice it to mention the advantages of performing *models* of biological processes, and – more generally – mathematicization of the whole of biology which, on this line, becomes an exact science. It is superfluous to enumerate examples of the great successes in this direction at many places of biophysical activity.

Besides the very valuable results produced in this field the mistakes should not be omitted. It ought to be emphasized that mathematical formulation often covers a lack of basic understanding: the following two examples demonstrate the fundamental mistakes of two excellent scientists. In his very interesting paper Eigen writes [6]:"

$$W_m^0 > E_{k \neq m}$$

(maximum selective value) (mean productivity in the not most favourable case)

gives a *physical definition* to the Darwinian fittest". Nobody can become wise by that.

The other example: Szent-Györgyi writes [7]: "If two particles are put together in a meaningful way then something new is born which is more than their sum: $1 + 1 > 2$. This is the most basic equation of biology." Such inequality, however, could be written – instead of the normal chemical equation $H + H = H_2$ – as $H + H = H_2 > 2H$, because hydrogen gas has gained new characteristics as compared with two hydrogen atoms (which have lost some characteristics).*

* In connection with Szent-Györgyi's opinion Eddington's remark should be quoted here [8]: "We often think that when we have completed our study of *one* we know all about *two*, because, "two" is 'one and one'. We forget that we have still to make a study of 'and'. Secondary physics (s. c. dealing with too improbable things p. 82) is the study of 'and' – that is to say, of organization." This conception of Eddington's—be it right or wrong—applies to the whole of natural science not only to biology.

Such "theoretical" expositions possibly further similar trials of younger biologists; thus, the other day I got a complimentary copy of a book entitled "The Principle of Life" [9] with the request to write my criticism to the author. That contained among others: "Your kind confidence inspirits me to express my conviction, based on the history of biology, that even small new facts often promote our science more than comments and theories." Furthermore, my criticism disapproved the fact that *assays* (born at desks) are nowadays overdone in comparison with *laboratory work*. Generally speaking besides such a splendid mental palace as Maxwell's theory, there are also hypotheses and theories which remind me of the story told me by a Russian colleague: somebody was appointed to a professorship at the German University in the Volga district to teach the students, mostly of German origin, the Russian language. The new professor addressed the students in his first lecture and intended to tell them that he would try to explain things not yet wholly understood by them. Instead of doing that, however, he, not being perfect in the German language, emphasized: "Ich werde versuchen, Ihnen dasjenige, was Sie bisher nicht vollständig verstanden haben, vollständing unverständlich zu machen". That sounds in English as follows: I shall try to make the things not yet completely understood by you totally ununderstandable. Of course, I did not wish to allude to certain persons or theories, and if, notwithstanding, I happened to hit the target, I quote the proverb: si non e vero, e bon trovato.

2.3. *Biocybernetics.* The new aspects of cybernetics found their way also into biology, and biocybernetics has already produced great successes and will achieve many more also in biophysics.

The most important result of this trend of thinking appears in considering every single living being as an *automaton*. According to this conception, one of the most important tasks in biophysical research is to find out the mechanism by which living systems coordinate their functions, i.e. how they bring their different processes under regulation or control. The first item in Hungary's Fifteen-Year Plan for biology including medical sciences sounds: "The Mechanism of Control of Life Processes."

Considering a living system as an automaton preserving its normal function by regulation or control one comes to the conclusion that our social medical organisation is wrong from a certain point of view. Namely, people immediately want outside help and use a lot of medicaments in the case of the smallest inconvenience because they are not properly educated and do not understand that their organism as an automaton is able to eliminate many temporary complaints by regulation, perhaps better than by intervention from outside.

There is no doubt about it that biology, while heading for the exact sciences, gets fundamental help from the biocybernetical results produced by biophysics. Considering, however, the present state of affairs in this field one encounters many difficulties originating in misunderstandings and even misconceptions. Thus, even one of the most basic notions, *information* seems to be used by different authors with different meanings. In my opinion "the lions roar" cannot be considered as information for antelopes (sc. to make them flee), it is a sign but not

a signal carrying information as a physicist, a good expert in acoustics, wrote erroneously in a paper sent to me. Similarly it seems to me that the equation [10]

$$1 \text{ bit} = 10^{-23} \text{ cal/Mol} \cdot \text{C}^\circ$$

is a misleading statement and a continuation of Maxwell's witty remark about the minidemon into witticism on the side of commentators.

Such and other mistakes in this field must, however, be ascribed not only to single individuals; they are much more deeply impregnated in our method of thinking. Thus, e.g. many authors dealing with living automatons assume some effort made by living systems to achieve an "optimum" of regulation, because engineers naturally try to produce the best possible results for the control of the automatons built by themselves.

A technician when controlling a closed loop of his automatic machine is fully justified in calling interference from outside – and of course not planned – a disturbing factor; in contrast to that e.g. the effect of a cerebral process on systolic blood pressure is of the same biological "rank" as the functions of other organs in the closed loop and not a disturbing factor. At any rate scientists are – without any affection for teleology – very much inclined to accept such "anthropocentric" thinking. Accordingly we learned, e.g. that the French astronomer Leverrier computing the aberrations in the orbit of Uranus described them as "disturbances" caused by another satellite. But only for the scientist Leverrier could Neptune play the role of a disturbing factor, objectively it is a member of the solar system similarly to all others.

If biologists keeping an open mind consider living systems and their functions as all other phenomena of nature without prejudice then biocybernetics will be one of the most important sections of biophysical research in biology heading for the exact sciences.

3. The prospects

Besides the general problems of biophysics dealt with so far there are some more special topics which, together with the former ones, in future should and will be worked out in biophysics.

3.1. Nowadays one more frequently encounters the expressions *bound water*, or *bound Na*, *K*, *Ca* (e.g. [1, 12, 13, 14]), mostly in affirmative but sometimes in negative formulation. I, personally, have taken an affirmative stand in this question, but cannot help pronouncing that the experimental data do not seem to prove unequivocally that these inorganic atoms are bound, i.e. their activities are less in certain biological milieus than in dilute solutions. The greatest difficulty in considering these inorganic atoms as bound in certain cellular regions seems to be in the circumstance that no exact explanation has so far been given for such a reaction.

Notwithstanding, some phsyical analogies of the affirmative standpoint can be mentioned [15], so e.g. the different behaviour of electrons when bound in an atom, and then their movement is quantized in contrast to their free trans-

lational movement. As another example, the neutron being intranuclear or free, could be given. Referring to these physical examples the problem could perhaps be formulated as follows: since the behaviour of e.g. potassium is quite different in certain cellular circumstances and in free solution the hypothesis that it is bound in the former case seems to be acceptable.

The question of *bound water*, on the other hand, is not a hypothesis, but an established fact. This difference in the cases of bound potassium and bound water lies in the differing methods of investigation. In both cases the decrease in activity should be demonstrated; but the living cells had doubtless suffered an injury by the microcathode stuck into the cell in the very region whose bound potassium was to be investigated. In contrast to that, the bound water e.g. of a frog muscle, i.e. the decrease of the vapour pressure of its water content, can be measured without any injury of the excized muscle, as was done in our institute and published in 1950 [11, 13]. The fact of bound water has been since then corroborated by many authors among them by those who once more discovered it, seemingly without knowing the earlier results and consequently not citing them.

3.2. *Water.* The question of "bound water" is but a small section of the large problem which could be named "*water in biology.*"

Dealing with that problem, one has to consider the history of the role played by water in the science of solution. When expounding this topic van't Hoff, the founder of physical chemistry, looked upon water in the case of a solution as upon vacuum in the case of a gas. Though this error has often been plainly corrected in many textbooks [e.g. 16, 17, 18] it still persists in the expression of osmotic pressure $p = cRT$, where c stands for the *concentration of the solute.* Furthermore, though these books plainly describe the difference in the activity of water as the decisive factor in the process of osmosis they still ascribe fundamental importance to the *semipermeability* of the membrane. According to this conception the difference in magnitude between the diffusing particles makes it possible for water molecules to migrate more easily through the membrane in con trast to solute particles.

Following this line we put the question in our laboratory what happened *when normal and heavy water* were on the two side of a "semipermeable" membrane. Both molecules are of the same magnitude [19], 2.76 Å, but the vapour pressure

of normal water at 20°C is 17.53 mmHg, and

of heavy water at 20°C is 14.97 mmHg.

We observed *migration of normal water into the heavy water* through the "semipermeable" membrane of $Cu_2Fe(CN)_6$, and measured either the increase in ml on the side of heavy water (a few millilitres), or the highest pressure against which this migration could proceed (e.g. 1 atm.). Thus besides the conception concerning the semipermeability or the concentration of the solute the fundamental importance of the difference in activity or vapour pressure on the two sides should be emphasized.

This exposition serves to substantiate the following argumentation describing as quite wrong the conception of the role played by water in biological systems. Namely, water is widely considered – according to van't Hoff's miscon-

ception – as *a passive medium* in which the functions of the macro- and microparticles take place inside the cell. In contrast to that *water is a part of the working units*, e.g. of hydrated proteins. Thus, the ingenious conception of Szent-Györgyi, concerning the electronic conductance of proteins is not affected by experiments on *dried* proteins because in living systems only *hydrated* proteins function. It is another question whether electrons or protons participate more in the electrical conductance of proteins.

Advancing on these lines one reaches even the problem whether the process of *excitation* is brought about chiefly by the migration of electrons or protons both being of the same dimension. Considering this question we are already discussing a section of *electron biology*, and thus a wide field opens for future research in biophysics then already fusing with biochemistry in many respects.

3.3. *Biofrequencies.* The wide field of electron biology will contain several districts; one of the most important ones appears to be the very interesting part dealing with *rhythmic phenomena in biology*. This material is also too extensive, and therefore the biochemical oscillations [20] will not be treated here, furthermore only those topics will be discussed for which the frequency $f \gg 1$ Hz. To this domain belong the spikes of excited nervous units.

The frequent spikes of excited nervous tissues have often been described [21, 22, 23] and attempts have been made to explain or model them by "rhythmic electrodes" in ionic solutions. Even Teorell [24] newly published such an interesting model (see also Prigogine, [25]).

What should specially be discussed this time is the phenomenon, described also during a century, according to which excitable tissues being *stimulated with direct current produce frequent spikes*. Thus Frey [26] made the very interesting remark in 1883: "Es muss also dem Nerven die Fähigkeit zugetheilt werden, den stetigen Verlauf des constanten Stromes in getrennte Erregungsstösse umzusetzen . . ." ("The ability should be ascribed to nerves to transform the constant course, of d.c. into separate impulses of excitation . . ."). A similar exposition was published by Biedermann [27] and also by Bethe [23] describing the fact that light stimulus was accompanied by direct current in the retina, but by frequent spikes in the fibres of the optic nerve. The same is observable in any process taking place in the sensory end-apparatuses and in the nerve fibres conducting the impulses from the former. It seems especially important that the same was found [28] in the case of generator- and action potentials on a single nerve fibre. As a continuation of this trend of thoughts the experimental results of Katz should be quoted ([3], p. 131): "It has recently been found that the frequency of this discharge can be enormously increased by passing a steady electric current through the terminal part of the motor nerve."

Summarizing these data one can formulate them as follows: *biological tissues are able to transform direct current into alternating current.* As an explanation I should like to propose the so-called *Gunn-effect* [29, 30, 31]. It consists, as is known, of the phenomenon discovered by Gunn in 1963 that a thin wafer of GaAs crystal produced high frequency alternating current when direct current of ~ 1000 Vcm^{-1} was applied to it. This very important fact has, as far as I

know, seemingly not found its way into the biologists' perception, and Gunn's name is missing from the papers of Teorell [24] in 1966, Hodgkin [31] in 1967 and even Prigogine [25] in 1971.

3.4. *Nuclear biology.* It is the wish to bind the future of biophysics much more to advances in physics that urges me to continue and finish this study expounding what is meant by this title.

The real meaning appears perhaps when its relation to molecular biology is taken into consideration. Trifling with the molecular aspect of natural processes as a contrast with the nuclear aspect one could describe the spatial relations as follows: if we consider the magnitude of a lecture room where the processes of a nucleus take place then the peripheral electrons belonging to the same nucleus which participate in chemical reactions should be located as far from it as e.g. Paris; that is why the chemical reactions could be looked upon as "superficial" in principle.

Speaking seriously, one can ascribe to the *biochemical* aspect the nearly generally accepted view – I should like to call it a dogma – that *isotopes* being chemically equal cannot be discriminated by living systems. It has, however, never been proved by anybody that bioentities have but chemical means at their disposal. Quite on the contrary, the discrimination of normal and heavy water by life processes is generally accepted and explained by their different diffusibilities due to their different masses, which is a physical and not a chemical aspect.

Similarly, radiobiology and especially nuclear medicine consider *the active and inactive isotopes* of an element as biologically identical in experiments performed with tracer technique. This view neglects the so-called isotope effects and also the possible biological consequences of radioactive processes. The consequences of radiation can directly manifest themselves in physical systems, e.g. ionization of the air due to radiation, living systems, however, show radiation effects only through a series of transmissions linked by consecutive biological reactions.

The phenomena of e.g. radiolysis or polymerization also calls our attention to the general question whether or not the radioactive isotopes in experiments with tracer technique really behave quite identically with the inactive isotopes of the same element. This question gets even more emphasis when the behaviour of an *active isotope*, injected *in free solution* into the body, is considered to be identical with that of the *inactive isotope* of the same element *organically bound* in the cell. Proceeding to *energetic problems* the well known fact is to be mentioned that nuclear processes are accompanied by energy changes about 10^6 times larger than the energy of a chemical reaction consisting in exchange of peripheral electrons and representing about $1-10$ ev. The nuclear process of e.g. $n = p + e^- + \tilde{\nu}$ shows a mass defect of the magnitude of an electron representing about half Mev. If physics in future considers atoms as having their nuclei not in a static state but in a dynamic equilibrium then quite a new prospect of bioenergetics opens up for biophysics following this course. And the prospect will widen out when biophysics enables itself to apply the science of elementary particles to biology.

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ANNOUNCEMENT OF AN IAEA SYMPOSIUM

Title:	Radioimmunoassay and Related Procedures in Clinical Medicine and Research
Date:	10—14 September 1973
Location:	Istanbul, Turkey
Organizers:	International Atomic Energy Agency, Kärntner Ring 11—13, A-1010 Vienna, Austria

Scientific Secretaries: Dr. E. J. Garcia and Dr. E. H. Belcher. Medical Applications Section

The programme of this Symposium, the second to be organized by the International Atomic Energy Agency in the subject field, will cover all aspects of radioimmunoassay and related procedures and their applications. Particular attention will be given to the *in vitro* determination of substances of clinical interest such as hormones, vitamins and drugs. Sections of the programme will be devoted to recently developed techniques for the detection of tumour, viral and bacterial antigens, and to assays employing labelled antibodies and naturally occurring specific receptors.

Further information, participation forms and forms for submission of a paper intended for presentation at the Symposium will be obtainable from national authorities for atomic energy matters. Abstracts of such papers must be submitted to the International Atomic Energy Agency through these authorities.

Mitteilungen der Gesellschaft für Biologische Chemie

18. Rundschreiben (Januar 1973)

Tagungsankündigungen

FEBS Advanced Course 24

8.—14. 3. 1973 in Hintermoos/Salzburg

Thema: *Protein/Nucleic Acid-Interaction*

Es werden die Protein-Nucleinsäure-Wechselwirkungen in folgenden Systemen behandelt: DNA- und RNA-Polymerasen, RNA-Replikasen, DNA-Modifikationen und Restriktionsenzyme, andere DNA-bindende Proteine, Repressoren, Nucleohistone, Aminoacyl-tRNA-Synthetasen, Ribosomen und Viren.

Unterbringung in der Bundespostschule Hintermoos. Teilnahme an einem Skikurs ist möglich.

Die Teilnahmegebühr beträgt 2000,— ÖS, alles eingeschlossen.

Die Frist für Bewerbungen ist am 1. 12. 1972 abgelaufen.

Information: Prof. Dr. U. Wintersberger, Institut für Krebsforschung der Universität Wien, Borschkegasse 8a, A-1090 Wien 9, Österreich.

Jahrestagung der Gesellschaft für Biologische Chemie,

zu der auch die Mitglieder der Schweizerischen Gesellschaft für Biochemie eingeladen werden.

Konstanz 12.—14. 3. 1973

Themen: 1. Struktur und Funktion von Oxidoreduktasen
2. Energiekonservierung und aktiver Transport
3. Biochemie des Muskels

Es sind keine Plenarvorträge, aber Round-Table-Diskussionen vorgesehen. Beitrag für Mitglieder DM 20,—, Studenten DM 5,—. Nach Eingang des Tagungsbeitrages wird das Programm zugeschickt.

Information: Prof. Dr. H. Sund, Univ. Konstanz, Fachbereich Biologie, D-775 Konstanz, Postfach 733.

Vom 15.—19. April 1973 findet in Dublin ein *FEBS Special Meeting on Industrial Aspects of Biochemistry* statt.

Hauptthemen: Enzymes; Biochemistry and the Environment; Pharmaceutical and Fine Chemicals; Biological Syntheses; Mode of Action of Drugs; Food and Bulk Materials; Analytical Techniques. Es ist auch eine *Ausstellung* von Instrumenten und Apparaten für Laboratorien und Industrie sowie von Chemikalien und Fachliteratur vorgesehen.

Information: FEBS Special Meeting, Secretariat, IMA Conference Centre, 10, Fitzwilliam Place, Dublin 2, Ireland.

XI. International Biochemical Meeting (XI Jornadas Bioquímicas Latinas)

24.—27. April 1973 in Salamanca

Symposia über Membranproteine, Wirkungsmechanismus der Steroidhormone, Struktur und Stoffwechsel der Glykoproteine.

Weitere Themen : Hydrolasen, Lipoproteine, Oligoelemente, zyklisches AMP, Enzyme in der klinischen Biochemie, Stoffwechselregulation.

Information: Prof. J. A. Cabezas, Departamento de Bioquímica, Facultad de Ciencias, Salamanca, Spanien.

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24. Mosbacher Kolloquium

26.—28. April 1973 in Mosbach/Baden

Thema: *Regulation von Transkription und Translation vornehmlich bei Eukaryonten*

Information: Prof. Dr. E. Bautz, Institut für Molekulare Genetik der Universität, D-69 Heidelberg, Berliner Str. 15.

Die Biochemische Gesellschaft der DDR veranstaltet ein Symposium mit dem Thema

Intrazellulärer Proteinabbau

7.—11. Mai 1973 in Schloß Reinhardsbrunn, Friedrichroda

Information: Prof. Dr. H. Hanson, DDR-402 Halle, Hollystr. 1, Physiologisch-Chemisches Institut.

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2. Europäische Histamin-Tagung

anläßlich des 70. Geburtstages von Prof. Dr. Dr. E. Werle

17. und 18. Mai 1973 in Marburg

Themen: Histaminstoffwechsel sowie Fragen der physiologischen und pathophysiologischen Funktion von Histamin im Gastro-Intestinaltrakt, Zentralnervensystem und bei immunbiologischen Prozessen.

Information: Prof. Dr. W. Lorenz, Chirurgische Universitätsklinik, Abt. für Experimentelle Chirurgie und Pathologische Biochemie, 355 Marburg, Robert-Koch-Str. 8.

Die *Gesellschaft für Biomedizinische Technik* lädt zur Teilnahme an ihrer *Jahrestagung* ein

24.—25. Mai 1973 in Erlangen

Die Anmeldefrist für Übersichtsreferate und Kurzvorträge über Originalarbeiten am 1. 2. 1973 bereits abgelaufen. Anmeldung zur Teilnahme bis spätestens 1. 4. 1973 an Dr.-Ing. R. Blæser, Department für Biomedizinische Technik, Universität Erlangen-Nürnberg, 852 Erlangen Turnstraße 5.

Die Tagungsgebühr (für Mitglieder DM 50,—, für Nichtmitglieder DM 85,—) enthält die Kosten für den ausführlichen Tagungsbericht mit den Manuskripten aller Vorträge.

Ein Internationales Symposium über *Purinstoffwechsel beim Menschen* wird vom 18.—22. 6. 1973 an der Universität Tel Aviv abgehalten.

Information: The Organizing Committee, International Symposium on Purine Metabolism in Man, P. O. Box 16271, Tel Aviv, Israel.

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3. Linderstrøm-Lang Konferenz über

Biochemical Control of Cell Growth

27.—30. Juni 1973 in Espoo bei Helsinki

Unterkunft und Verpflegung werden ca. $60,— kosten. Es wird kein Teilnehmerbeitrag erhoben. Zuschüsse für die Reisekosten stehen in begrenztem Umfang zur Verfügung. Bewerbungen sind bis spätestens 15. April 1973 zu richten an Dr. J. Jänne, Department of Medical Chemistry, University of Helsinki, SF-00170 Helsinki 17, Finland.

Der IX. Internationale Kongreß für Biochemie findet vom 1.—7. 7. 1973 in Stockholm statt.

Information: Secretariat, IX. Internat. Congress of Biochemistry, c/o Svenska Kemistsamfundet, Wenner-Gren Center. 6 tr, S—11346 Stockholm.

Bitte beachten Sie in diesem Zusammenhang das mit diesem Rundschreiben an alle Mitglieder gesandte Angebot der Reiseorganisation World Travel für vergünstigte IT-Reisen nach Stockholm.

FEBS Advanced Cours 25 in Prag: 9.—13. 7. 1973 siehe FEBS Bulletin.

Das Department of Biochemistry der Universität Surrey und die Society of Chemical Industry veranstalten einen Residential Course über

Enzyme and Fermentation Biotechnology

16.—20. Juli 1973. in Guildford, Surrey, U. K.

Kursgebühr inkl. Unterkunft und Verpflegung £ 50.00.

Information: Dr. A. Wiseman, Department of Biochemistry, University of Surrey, Guildford, Surrey.

XVIth International Conference on the Biochemistry of Lipids

(Biochemistry of inositol lipids; control of fatty acid synthesis and related metabolism)

17.—20. Juli 1973, Nottingham, U. K.

Information: Dr. D. N. Brindley, Department of Biochemistry, University of Nottingham Medical School, Nottingham NG7 2RD, England.

Der 3. *EMBO-Course über Mikromethoden in der Molekularbiologie*

findet statt vom 17.—29. September 1973

im Max-Planck-Institut für Experimentelle Medizin, Göttingen. Der Kursus konzentriert sich auf praktische Übungen zur Handhabung von Mikro-Disk-, Mikro-Gradientengel- und Mikro-SDS-Gelelektrophorese; Mikro-Isoelektrische Fokussierung; Mikro-Immunopräzipitation; Mikro-Fingerprint-Analyse von dansylierten Aminosäuren; Mikro-RNA-Basenanalyse; Cytophotometrie und Cytofluorometrie von isolierten Zellen, zusammen mit anderen Methoden.

Kursgebühr DM 200,—, Unterkunft mit Verpflegung ca. DM 350,—. Eine beschränkte Anzahl von Stipendien ist erhältlich.

Information und Anmeldung: Prof. Dr. V. Neuhoff, Max-Plank-Institut für Experimentelle Medizin, 34 Göttingen, Herman-Rein-Str. 3.

Anmeldeschluß: 30. 4. 1973.

Gemeinsame Herbsttagung der Gesellschaft für Biologische Chemie und der Österreichischen Biochemischen Gesellschaft

3.—5. Oktober 1973 in Innsbruck

Themen:

1. Biochemie der höheren Pflanzen
2. Molekulare Aspekte des Wachstums und der Differenzierung
3. Biochemie der Viren einschließlich der Phagen
4. Regulation des Kohlenhydratstoffwechsels

Information: Prof. Dr. W. Sachsenmaier, Institut für Biochemie und Experimentelle Krebsforschung der Universität Innsbruck, A-6020 Innsbruck, Peter-Mayr-Straße 2.

Das Institut für Gärungsgewerbe und Biotechnologie veranstaltet das

3. Symposium Technische Mikrobiologie

30. Oktober bis 2. November 1973, Kongreßhalle Berlin

Wissenschaftliches Programm: Mikrobiologische Grundlagen (Cytologie, Systematik, Genetik, Selektion von Organismen); Mikrobieller Stoffwechsel (Stoffumwandlung, Produktbildung, Stoffwechsel-Regulation, Kinetik); Mikrobiologische und biochemische Verfahrenstechnik (Reaktortypen und ihr Verhalten, Ausstattung für Fermentation und "unit operations"

Meß- und Regeltechnik, Massen- und Wärmetransfer); Spezielle Prozesse (Zellsubstanzsynthese, primäre und sekundäre Metabolite, Enzyme, Lebensmittel, biologische Abbaureaktionen); Analytische Techniken.

Vortragsanmeldung: Anmeldeschluß 1. März 1973.

Information: Institut für Gärungsgewerbe und Biotechnologie, 3. Symposium 1973, 1 Berlin 65, Seestraße 13.

International Symposium on Wound Healing

8.—12. April 1974 in Rotterdam.

Information: The Secretariat, c/o Holland Organizing Centre, 16, Lange Voorhout, The Hague, The Netherlands.

Die *108. Versammlung der Gesellschaft Deutscher Naturforscher und Ärzte* wird vom 15.—19. September 1974 in der Kongreßhalle Berlin stattfinden.

Preisausschreiben

Preis "Biochemische Analytik"

Boehringer Mannheim GmbH hat den mit DM 10 000,— dotierten Preis gestiftet; er wird für hervorragende Arbeiten auf dem Gebiet der biochemischen Analytik alle zwei Jahre anläßlich der Tagung "Biochemische Analytik" in München vergeben. Für die Verleihung während der Tagung 1974 (23. bis 26. April) können jeweils eine Arbeit oder mehrere Arbeiten über ein Thema, die in der Zeit zwischen dem 1. Oktober 1971 und dem 30. September 1973 publiziert oder zur Publikation angenommen sein müssen, bis spätestens 15. November 1973 in dreifacher Ausfertigung eingereicht werden an:

Prof. Dr. Dr. Ivar Trautschold, Sekretär für den Preis "Biochemische Analytik", Medizinische Hochschule Hannover, 3 Hannover-Kleefeld, Karl-Wiechert-Allee 9.

Deutsche Gesellschaft für Endokrinologie

Preisausschreibungen für 1974

Preise	Schoeller-Junkmann-Preis	Marius-Tausk-Förderpreis
Dotierung	DM 15 000,—	DM 15 000,—
Stifter	Schering AG, Berlin	Organon GmbH, München
Bewerber	müssen in Europa ansässig und nicht älter als 40 Jahre sein	müssen in Europa ansässig und nicht älter als 33 Jahre sein
Gebiete	gesamte Endokrinologie (außer Diabetes mellitus)	klinische und klinisch-experimentelle Endokrinologie (außer Diabetes mellitus)

Die Bewerber werden gebeten, das Manuskript einer bisher unveröffentlichten Arbeit in zweifacher Ausfertigung in deutscher oder englischer Sprache bis zum 15. 10. 1973 an den Präsidenten der Deutschen Gesellschaft für Endokrinologie für 1973/74, Herrn Prof. Dr. med. J. R. Bierich, 74 Tübingen, Universitäts-Kinderklinik, Rümelinstraße 23, sowie einen kurzen Lebenslauf mit ihrem wissenschaftlichen Werdegang einzureichen. Nach Bestätigung des Manuskript-Eingangs durch die Deutsche Gesellschaft für Endokrinologie kann der Autor die Arbeit einer Zeitschrift zur Veröffentlichung einreichen.

Die Satzungen für die Preise sind beim Präsidenten der Gesellschaft erhältlich. Die Verleihung der Preise erfolgt auf dem 20. Symposium der Deutschen Gesellschaft für Endokrinologie, 1974.

Unilever European Fellowships 1973—1974

Up to three Fellowships will be awarded for the academic year 1973—74, two for research in biochemistry in a laboratory in continental Europe by British citizens resident in Great Britain and the other for work in Britain by European nationals. Applicants should hold a Ph. D. degree or offer evidence of having equivalent qualifications or experience. The Fellowships may be up to £ 1,700, but in special circumstances may exceed this figure.

The Fellowships are tenable for one year in the first instance and are available for work in any laboratory or institute in Britain or continental Europe, including Unilever Research Laboratories.

Travelling expenses will be paid and financial assistance for attendance at meetings in Europe may also be provided. The Fellowships shall be acknowledged in any publication resulting from the work carried out during the period of the award by a statement that the recipient was a "Unilever European Fellow of The Biochemical Society".

Applications, which should include an outline of the proposed research work, a statement that the applicant can be accepted at the chosen place of work, a list of the applicant's publications, and the names and addresses of two referees, are now invited. Application forms may be obtained from the Executive Secretary, The Biochemical Society, 7 Warwick Court, London, WC1R 5DP, and should be returned to him not later than 31 December 1972.

Mitgliedsbeitrag 1973

Der Mitgliedsbeitrag für 1973 beträgt unverändert DM 20,—, für Studenten DM 5,—. Darin ist der Mitgliedsbeitrag zu FEBS enthalten. Die Zahlung war am 1. Januar 1973 fällig. Eine besondere Rechnung ergeht nicht; der Eingang des Beitrages wird durch Übersendung der Mitgliedskarte quittiert.

Die Einziehung ausländischer Schecks, auch wenn sie auf DM-Beträge ausgestellt sind, ist mit relativ hohen Bankgebühren belastet. Bitte zahlen Sie deshalb in solchen Fällen DM 5,— zusätzlich, also DM 25,—.

Konten der Gesellschaft: Postscheckkonto Frankfurt (Main) Nr. 56909, Bankkonto Dresdner Bank AG, Wuppertal-Elberfeld, Nr. 5 049 714.

Bitte prüfen Sie, ob bei Ihrem Dauerauftrag die alte Bankkontonummer (713 715) in die neue Nummer 5 049 714 umgeändert ist.

Anschriftenänderungen

Der Schriftführer der Gesellschaft für Biologische Chemie, Prof. Gibian, D-1 Berlin 65, Müllerstraße 170/172 bittet dringend alle Mitglieder um sofortige Benachrichtigung bei Adressenänderungen, da sonst ein einwandfreier Informationsdienst nicht gewährleistet ist.

Hoppe-Seyler's Zeitschrift für Physiologische Chemie

Editors in Chief

A. Butenandt · F. Lynen · G. Weitzel

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