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Acetate Activation in Chlamydomonas reinhardti

I. Partial Purification and Characterization of Acetate Kinase

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(Received August 10, 1967)

1. Acetate kinase (ATP: acetate phosphotransferase, EC 4.7.2.1.) has been detected and purified from the fresh-water alga Chlamydomonas reinhardti cultured in the light with carbon dioxide.

2. The Mg^{2+} complex of adenosine-5′-triphosphate appears to be the active form of substrate. The Michaelis constant of the enzyme for $Mg-ATP^{2-}$ is 2.0 mM and for acetate 100 mM. The enzyme is specific for acetate, although it reacts to a small extent with propionate also.

3. Double reciprocal plots of initial velocity *vs* ATP concentration at different levels of acetate yielded linear but parallel curves. A similar kinetic pattern was obtained when the concentration of acetate was varied and that of ATP kept constant. The results of the initial rate experiments are compatible with the "Ping-Pong" mechanism. It is concluded that the mechanism of the acetate kinase studied appears to be different from that suggested for the bacterial acetate kinase.

Introduction

In most living cells the "active acetate" is formed by the pyruvate dehydrogenase complex (Reed, Cox, 1966). In microorganisms and plants two enzymes, acetate kinase (EC 4.7.2.1.) and acetate thiokinase (EC 6.2.1.1.) were shown to activate acetate directly. Among fresh-water algae there is a taxonomically heterogeneous group, the acetate flagellates, which cannot use carbohydrates as carbon source under heterotropic growth conditions but can use acetate and other fatty acids (Hutner, Provasoli, 1951). Chlamydomonas reinhardti belongs to this group.

This paper reports the detection and partial purification of acetate kinase from cell-free extracts of Chlamydomonas reinhardti cultured in the light with carbon dioxide, and describes some properties of the enzyme.

Abbreviations: ATP, adenosine-5′-triphosphate; ADP, adenosine-5′-diphosphate; AMP, adenosine-5′-phosphate; P, inorganic phosphate; PP, pyrophosphate; PCMB, p-chloromercuribenzoate.

Materials and Methods

Chemicals

The inorganic chemicals used were of A.R. grade. Adenosine-5′-triphosphate and adenosine-5′-diphosphate were obtained from Reanal, Budapest. PCMB was purchased from British Drug Houses, Ltd., London. Dowex 2×8 anion-exchange resin was obtained from Fluka A.G., Buchs.

Organisms and growth conditions

Wild-type *plus* and *minus* mating-type strains, Nos 89 and 90 of Chlamydomonas reinhardti were kindly supplied by the Culture Collection of Algae, Department of Botany, Indiana University, Bloomington. Experimental and stock cultures of the minus mating-type strain were grown as described previously (Staub, Dénes, 1966). The centrifuged cells were stored at —20°C.

Preparation of acetone powder

The frozen alga cells were thawed and acetone-dry powder was prepared as described previously (Südi, Dénes, 1967). When stored at 2°, the acetone-dry powder showed no loss in acetate kinase activity after several months.

Enzyme assay

The activity of the enzyme was determined by the hydroxamic acid method (Lipmann, Tuttle, 1945). For adjusting the pH of stock solutions (except hydroxylamine) potassium hydroxide was used. The pH of the hydroxylamine hydrochloride stock solution was adjusted to the required value with Tris base, and no additional buffer was required for stabilizing the pH. The "standard" reaction mixture for the determination of enzyme activity contained 200 μmoles of Tris base, 200 μmoles of hydroxylamine hydrochloride, 400 μmoles of potassium acetate, 20 μmoles of ATP, 20 μmoles of magnesium chloride and the enzyme in a total volume of 2.0 ml. The pH of the mixture was 7.0. After incubation at 37°C the reaction was arrested by the addition of 2.1 ml of ferric chloride reagent, consisting of 0.7 ml of 12% trichloroacetic acid, 0.7 ml of 3 N hydrochloric acid and 0.7 ml of 5% ferric chloride dissolved in 0.1 N hydrochloric acid. After centrifugation, the absorbance of the acethydroxamic acid—ferric complex was determined at 540 mμ in a Spectromom 360 spectrophotometer. One unit is defined as the amount of enzyme catalyzing the formation of 1.0 μmole of acethydroxamic acid per minute. Specific activity is expressed as units per mg of protein.

Miscellaneous determinations

Protein was determined by the method of Lowry et al. using crystalline bovine serum albumin as a standard (Lowry et al., 1951). Inorganic phosphate was measured colorimetrically (Fiske, SubbaRow, 1925). The determination of ATP, ADP and AMP was based on the adenine content of the nucleotides as determined by the absorbance at 260 mμ after separating them from each other by ion-exchange chromatography (Cohn, Carter, 1950).

Results and Discussion

Preparation of the enzyme

Step 1. Preparation of the crude extract. 10 grams of acetone-dry powder of the alga cells and 100 ml of 0.05 M Tris-HCl buffer (pH 7.5) containing 0.05 M potassium chloride were ground at 0°C in a porcelain mortar to a homogeneous paste with a porcelain pestle. After 30 minutes the suspension was centrifuged at 25 000 g and the greenish-yellow supernatant (55 ml) used for further purification of the enzyme.

Step 2. Fractionation with $(NH_4)_2SO_4$. The crude extract was fractionated with solid ammonium sulfate at 0°C between the limits of 0 and 0.25, 0.25 and 0.40 saturation. The first fraction which had little or no activity was discarded. The precipitate of the second fraction containing the enzyme was dissolved in 0.1 M Tris-HCl buffer (pH 7.5).

Step 3. Fractionation with acetone. The enzyme solution from Step 2 was brought to 50% acetone concentration by volume with rapid stirring and dropwise addition of acetone cooled to —15°C. The inactive precipitate was removed by centrifugation at —10°C. The clear supernatant fluid was then brought to 60 per cent acetone concentration as before. The precipitate was centrifuged and dissolved in 0.1 M Tris-HCl buffer (pH 7.5). A summary of the purification procedure is shown in Table 1.

Table 1

Purification of acetate kinase

(10 g of dry cells)

Step	Fraction	Volume, ml	Activity, units/ml	Protein, mg/ml	Specific activity	Yield, %
1	Crude extract	55	0.8	10	0.08	100
2	Ammonium sulfate fractionation	33	1.0	8	0.125	75
3	Acetone fractionation	10	2.1	1.5	1.4	48

The nature of acetate activating enzyme in C. reinhardti

Two different mechanisms are known for the activation of acetate. One of these is found in animal tissues (Nachmanson, Machado, 1943; Lipmann, 1945; Chou, Lipmann, 1952; Beinert et al., 1953), in yeast (Lipmann et al., 1952; Jones et al., 1953; Berg, 1956), in plants (Millerd, Bonner, 1954) and in Rhodospirillium rubrum (Eisenberg, 1955). In these organisms a single enzyme referred to as acetate thiokinase (EC 6.2.1.1.) catalyzes the reversible conversion of ATP acetate and CoA to AMP, PP and acetyl-CoA.

$$\text{ATP} + \text{acetate} + \text{CoA} \rightleftharpoons \text{AMP} + \text{PP} + \text{acetyl-CoA} \qquad (1)$$

In the presence of hydroxylamine and absence of CoA the same enzyme catalyzes the following reaction:

$$\text{ATP} + \text{acetate} + \text{NH}_2\text{OH} \rightarrow \text{AMP} + \text{PP} + \text{acethydroxamate} \qquad (2)$$

The other mechanism found so far only in bacteria is initiated by the phosphorylation of acetate with ATP by acetokinase (EC 4.7.2.1.) (Lipmann, 1944, 1954; Barker, 1951; Stern, Ochoa, 1951; Rose et al., 1954).

$$\text{ATP} + \text{acetate} \rightleftharpoons \text{acetylphosphate} + \text{ADP} \qquad (3)$$

In the presence of hydroxylamine

$$\text{ATP} + \text{acetate} + \text{NH}_2\text{OH} \rightarrow \text{acethydroxamate} + \text{ADP} + \text{P} \qquad (4)$$

It is possible to distinguish between the two possible mechanisms of acetate activation by determination of the nature of compounds produced by the enzymatic reaction (Equations 1 and 3). In the presence of ATP, acetate and hydroxyl-

Table 2

Time course and stoichiometry of acetate activation catalyzed by acetate kinase from C. reinhardti

The standard reaction mixture was used containing 0.45 mg of protein per ml. The reaction was arrested by heating the mixture to 100°C for 1 min. Determinations as described under Methods

Reaction time, minutes	Products formed, μmoles		
	acet-hydroxamate	ADP	P_i
5	1.09	—	1.15
10	2.1	—	2.1
15	3.0	3.1	—
30	5.9	6.2	—

amine acetate thiokinase catalyzes the formation of AMP, PP and acethydroxamate (Equation 2), while acetokinase catalyzes the formation of ADP, P and acethydroxamate. As shown in Table 2, the acetate activating enzyme extracted from C. reinhardti catalyzes the stoichiometric formation of ADP, P and acethydroxamate from ATP and acetate, according to Equation 4. We could not detect any acetate thiokinase activity in the cell-free extract of C. reinhardti cultured in the light with carbon dioxide as carbon source.

pH dependence, specificity and activating-ion requirement

The acetate kinase has a broad pH optimum between 6.8 and 7.5 as shown in Fig. 1. The fatty acid substrate specificity of the enzyme is very narrow, it is restricted to acetate and propionate. The rate of the reaction with propionate is only about one-fourth of that with acetate. The apparent K_m values of the

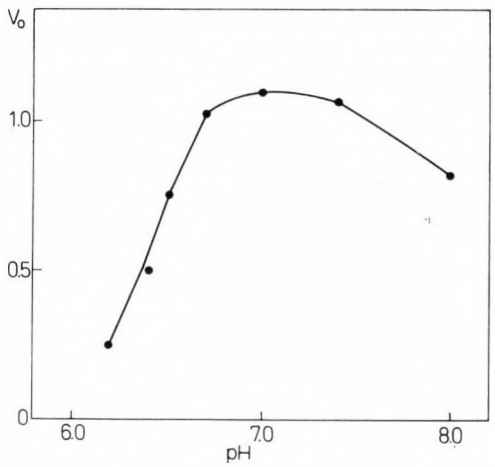

Fig. 1. pH-dependence of the activity of acetate kinase. The standard reaction mixture used contained 0.3 mg/ml of protein and Tris-buffer. Initial velocity v_0 is expressed as μmoles of acethydroxamate formed in 20 min

enzyme for acetate and for propionate were obtained from Lineweaver-Burk plots and are 100 mM for acetate and 200 mM for propionate. The enzyme is specific for ATP in the forward reaction like the acetate kinase purified from bacteria (Rose et al., 1954). The enzyme has an absolute requirement for bivalent ions and has no activity in the absence of added ions. As shown in Table 3, Mg^{2+} and Co^{2+} are the most stimulatory, Mn^{2+} and Zn^{2+} are less effective. In contrast to the bacterial acetate kinase, PCMB only slightly inhibits the activity of the enzyme purified from the alga cells.

Table 3

Specificity, requirement for activating ions and inhibition of acetate kinase

The standard reaction mixture containing 0.25 mg of protein was used except for the compound tested. The mixtures were incubated at 37°C for 20 minutes and the reaction was arrested by the addition of hydroxamic acid reagent as described under Methods. In Experiment A the activity of the enzyme was assayed in the presence of half-saturating concentrations of acetate or propionate. In Experiment B the effect of activating ions was tested. In Experiment C the enzyme was incubated at 25°C for 30 minutes with PCMB and the residual activity was determined

Experiment	Compound tested	Conc. M	Activity
A	acetate	0.1	2.50
	propionate	0.2	0.63
B	none	—	0.00
	Mg^{2+}	0.01	2.50
	Co^{2+}	0.01	2.55
	Mn^{2+}	0.01	1.80
	Zn^{2+}	0.01	0.75
C	PCMB	0.0	2.50
	,,	10^{-7}	2.25
	,,	10^{-5}	1.75
	,,	10^{-3}	1.75

Kinetic analysis of the mechanism of enzyme action

We have observed that the activating effect of Mg^{2+} depends on the concentration of ATP. Maximum activity was obtained when the ATP/Mg^{2+} ratio was equal to 1. Excess ATP inhibited the reaction. On the basis of these observations it seems probable that the substrate of the enzyme is not ATP in itself but the Mg–ATP^{2-} complex. Since the stability constant of Mg–ATP^{2-} for the reaction

$$Mg\text{–}ATP^{2-} \rightleftharpoons Mg^{2+} + ATP^{4-} \qquad (5)$$

is 20 mM (O'Sullivan, Perrin, 1961) it is possible to calculate the concentration of Mg–ATP^{2-}, ATP^{4-} and Mg^{2+} in each experiment (Melchior and Melchior, 1958). The Lineweaver-Burk plot of the reciprocal of the initial velocities against the reciprocal of the concentration of ATP, where ATP and magnesium chloride were added at equimolar concentrations, is not linear, as shown in Fig. 2. The plot becomes linear only, when the variable substrate is considered to be Mg–ATP^{2-}, as described previously for galactokinase (Ballard, 1966). It has been shown earlier that information on the mechanism of two-substrate enzymic reactions may be obtained from the analysis of experimental Lineweaver-Burk plots

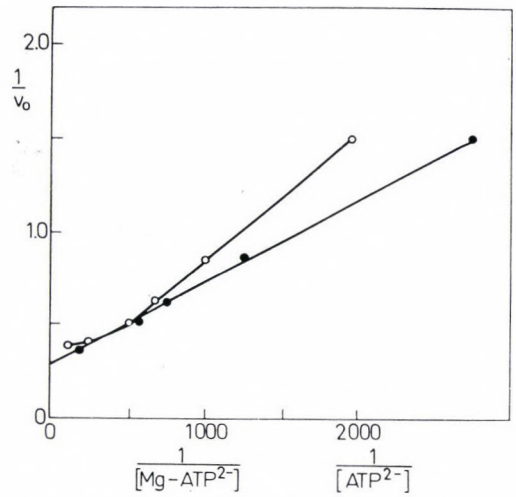

Fig. 2. Effect of ATP concentration on the initial velocity of acetate kinase. The concentrations of ATP and of magnesium are the same. The concentration of Mg—ATP^{-2} was calculated by using the stability constant described in the text. The standard reaction mixture was used except for Mg^{2+} and ATP; ○ — ○ 1/ATP; ; ● — ● 1/Mg—ATP^{2-}

produced by keeping the concentration of one substrate at several constant levels and determining initial velocities when the concentration of the second substrate is varied (Segal et al., 1952). The Lineweaver-Burk plots obtained are linear

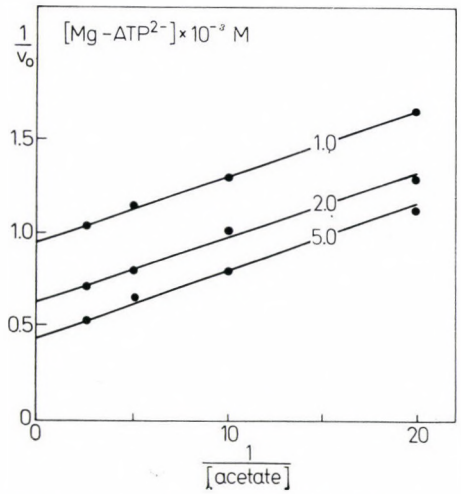

Fig. 3a. Double reciprocal plot of initial velocities (v_0) against acetate concentration in a series of fixed concentrations of Mg—ATP^{2-}. The standard reaction mixture was used except for the substrates tested

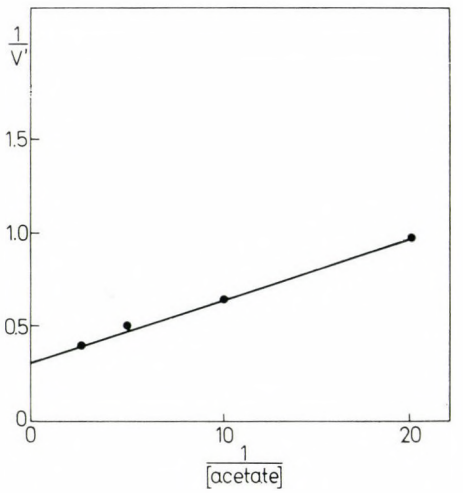

Fig. 3b. Secondary plot from the data of Fig. 3a for the determination of K_m for acetate

within the limits of experimental errors and over the concentration ranges as shown in Figs 3a and 4a. The lines are parallel and no apparent convergence to a common point is evident. The intercepts on the ordinate are the reciprocals of the apparent maximal velocities, $1/v'$, and the intercepts on the abscissa are negative reciprocals of apparent Michaelis constants $1/K'_m$. The Michaelis con-

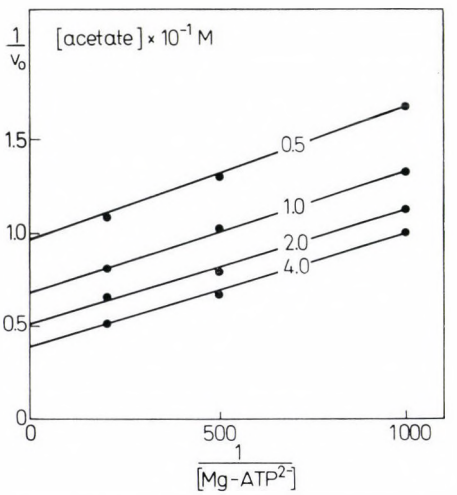

Fig. 4a. Double reciprocal plot of initial velocity (v_0) against $Mg-ATP^{2-}$ concentration in a series of fixed concentrations of acetate. The standard reaction mixture was used except for the substrates tested

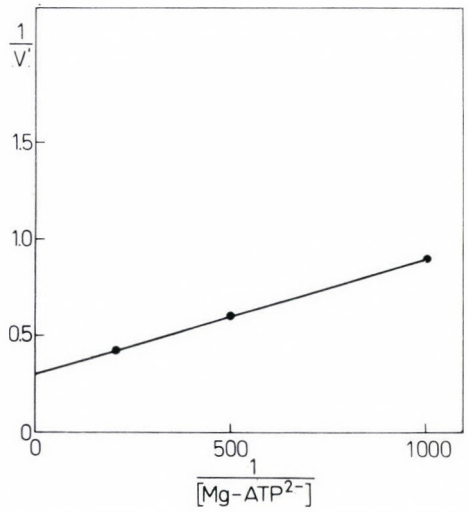

Fig. 4b. Secondary plot from the data of Fig. 4a for the determination of K_m for $Mg-ATP^{2-}$

stants of the enzyme for acetate (K_{ac}) and for $Mg-ATP^{2-}$ (K_{Mg-ATP}) obtained graphically from the secondary plots of the values of $1/v$, obtained in Figs 3a and 4a against the reciprocal of the concentration of acetate and $Mg-ATP^{2-}$ are 100 mM for acetate and 2.0 mM for $Mg-ATP^{2-}$. According to the initial velocity data presented, the mechanism of the reaction appears to be Ping-Pong (Cleland, 1963). Characteristics of the Ping-Pong mechanism are that the enzyme reacts with the first substrate and a product is released before the addition of the second substrate. The standard free energy change of the reaction catalyzed by acetate kinase (Lipmann, 1944) and the chemical nature of the substrates and of products permit only the following mechanism, proposed originally by Boyer and Harrison (1954)

$$E + ATP \rightleftharpoons E \sim P + ADP \tag{6}$$

$$E \sim P + \text{acetate} \rightleftharpoons E + \text{acetylphosphate} \tag{7}$$

The mechanism of action of acetate kinase purified from Escherichia coli seems to be different and appears to involve the random or sequential formation of a ternary complex before the products are released (Rose et al., 1954). It is known that the structure or mechanism of action of an enzyme may be different according to its origin. A similar difference as in the case of acetate kinases was observed in the mechanism of action of hexokinases (EC 2.7.1.1.). As to the hexokinase

purified from rat brain or muscle it was found that the mechanism of action of these enzymes is Ping-Pong (Hanson, Fromm, 1965). On the contrary, the mechanism of action of yeast hexokinase seems to involve the random formation of a ternary complex before release of the products occurs (Zewe et al., 1964).

Further studies on the acetate activation in C. reinhardti are in progress.

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The Mechanism of Ion Transport in Human Erythrocytes

II. The Role of Histamine in the Regulation of Cation Transport

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(Received August 11, 1967)

"Downhill" potassium outflow in human erythrocytes caused by the addition of NaF or IA* + purine nucleosides, respectively, is accompanied by an uptake of hydrogen ions. The rate of potassium/hydrogen ion exchange is always enhanced in the presence of histamine. Calcium ions and NADH are required for this transport process. A further increase in the rate of cation transport is observed when oxidative deamination of histamine is inhibited. On the other hand, the rate of ionic movement is markedly diminished in the presence of antihistamines or DNFB. Our results show that the "downhill" cation transport of erythrocytes is also subjected to metabolic regulation and that histamine is probably one of the compounds that may play a definite role in this process.

Introduction

Preservation of the unequal distribution of potassium and sodium ions in red blood cells and the surrounding plasma is a result of intricate physicochemical and biochemical processes. Transport processes realized against an electrochemical potential gradient (so-called "uphill" transport phenomena) require by all means active cellular functions and an appropriate source of energy. Energy is supplied by the high energy phosphate bonds of ATP (Straub, 1953; Gárdos, 1954), while the transformation of energy into ionic movements is carried out with the aid of the "transport ATPase" system localized in the cell membrane (Post et al., 1960; Dunham, Glynn, 1961; Gárdos, 1964).

Transport processes which have the same orientation as the electrochemical potential gradient ("downhill" processes) are seemingly of a passive character, and do not require energy. This concept is, however, not correct. The rate of cation movements is subjected to metabolic control in the cases of downhill transport as well.

* Abbreviations: ATP = adenosine 5′-triphosphate; NAD = nicotinamide-adenine dinucleotide; NADH = reduced nicotinamide-adenine dinucleotide; 2,3-DPG = 2,3-diphosphoglycerate; ATPase = adenosine triphosphatase; NADase = nicotinamide-adenine dinucleotidase; IA = iodoacetate (sodium salt); EDTA = ethylenediaminetetraacetate; EGTA = ethyleneglycol bis-(β-aminoethylether)-N,N′-tetraacetate; DNFB = 2,4-dinitrofluorobenzene.

Inhibition of glycolysis by various inhibitors or lack of substrate results in a breakdown of ATP. As a consequence, active cation transport will cease, an outflow of potassium ions will begin, and sodium ions will enter the cells at the same time. The rate of this ion transport is comparatively small, 1.5—2.0 meqs/l erythrocytes/hour, but in some cases, e.g. in the presence of lead ions, NaF, or IA + purine nucleosides, a high rate potassium outflow of 5—20 meqs/l erythrocytes/hour has been observed (Passow, Tillmann, 1955; Gárdos, Straub, 1957; Gárdos, 1956, 1960). The main characteristic of this artificially produced potassium outflow is that it is not accompanied by an equimolar sodium influx but, instead, an uptake of hydrogen ions takes place in the biological systems (Dunker, Passow, 1950; Szőnyi, 1960; Passow, 1961).

It has been stated in previous investigations that the presence of free calcium ions in the blood plasma was a prerequisite of high rate potassium outflow (Gárdos, 1958a, 1958b, 1959, 1961). Removal of calcium ions either by washings or in the presence of chelating agents led to the termination of this transport process. Further it has been shown that the rate of potassium outflow is regulated by the 2,3-DPG metabolism of the red blood cells (Gárdos, 1966a, 1966b, 1967). If the enzyme reactions of the 2,3-DPG shunt are activated, phosphoglycerate kinase is inhibited and at the same time ion transport is blocked.

The rate of potassium outflow may be influenced by several N-containing compounds, e.g. NH_4Cl, hydrazine, histidine and histamine (Passow, 1961, 1963). It is known that histamine — although at low concentrations — is a standard component of blood plasma. Further, it is also a well-known fact that in the state of clinical shock the level of plasma histamine may rise 100- to 200-fold compared to its original level. The possibility arises that histamine itself, or one of its metabolic products might be the physiological regulatory substance of the rate of downhill transport processes in blood. Based on these assumptions experiments have been carried out to study the changes in permeability under the effect of histamine. The most important results of these investigations are presented in this paper.

Methods

Fresh, defibrinated human blood was used in the *in vitro* experiments at 37 °C. Samples contained 75 per cent of normal blood; substrates, inhibitors and activators were present in the remaining 25 volume per cent. When working with washed erythrocytes, the blood was spun for 10 min at 3000 r.p.m., the serum and the buffy coat were removed and the cells were washed twice with three volumes of 166 mM NaCl.

Potassium and sodium were determined by flame photometry in the deproteinized extracts of whole blood or sera, respectively. Cell potassium contents were calculated from the data of flame photometry and the actual

hematocrit values. Radioactivity determinations of ^{42}K were carried out using a well-type scintillation crystal detector. Hydrogen ion uptake of cells was registered manometrically by the Warburg techniques as described by Szőnyi (1960) in a 95 per cent N_2—5 per cent CO_2 atmosphere. ATP determinations were performed enzymatically according to the Boehringer test, with the aid of phosphoglyceraldehyde-dehydrogenase and phosphoglycerate kinase (Bücher, 1947). Lactic acid was assayed with the method of Barker and Summerson (1941), inorganic phosphate and ribose were determined colorimetrically by the method of Fiske and SubbaRow (1925) and Mejbaum (1939), respectively.

Results

Effect of histamine on ion transport in normal blood

In cases, when carbohydrate metabolism and ATP levels were normal, histamine did not affect the transport of potassium and sodium ions. No differences were observed with respect to either net ion movements or active potassium transport (determined by ^{42}K exchange). Histamine had no effect on carbohydrate metabolism either (lactate formation, inorganic phosphate and ATP levels). If — instead of glucose — inosine was chosen as substrate in the blood, the presence of histamine was similarly irrelevant with respect to both carbohydrate metabolism and cation transport.

Effect of histamine on ion transport in the presence of glycolytic inhibitors

The effect of histamine at different concentrations was investigated in the presence of IA + inosine. The maximum effect of histamine to increase permeability was found to occur at a concentration of 0.5 mM histamine (Table 1).

Table 1

The effect of histamine at different concentrations on potassium outflow in human erythrocytes in the presence of 1 mM IA + 2.5 mM inosine

Final concentrations of histamine in mM	K^+ loss ΔK^+/120 min μmoles/ml cells
—	9.0
0.05	16.0
0.10	21.0
0.50	25.0
1.00	23.0
2.50	20.0
5.00	13.0
10.00	9.0

The effect was less pronounced at concentrations below or above this value. When the amount of histamine was raised to 10 mM it had no effect at all on permeability. These data are in line with Passow's (1963) results obtained under different experimental conditions.

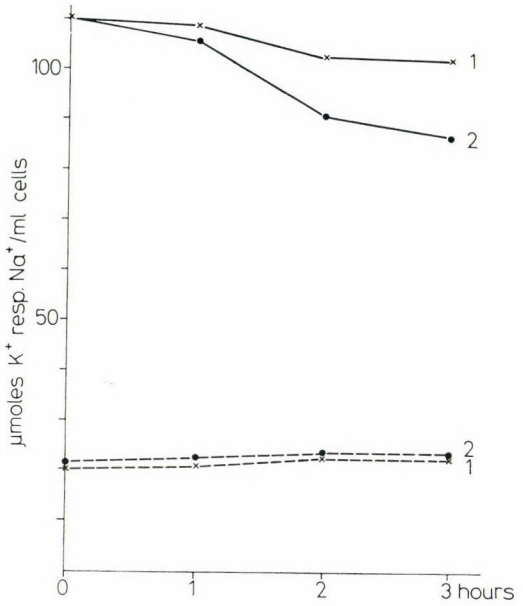

Fig. 1. Effect of histamine on the kinetics of net potassium and sodium ion transport in human erythrocytes in the presence of 8 mM NaF. 1: control; 2: 0.5 mM histamine. The solid lines indicate potassium ion transport, the broken lines mark sodium ion transport

The effect of 0.5 mM histamine on potassium and sodium ion transport was also tested using various glycolytic inhibitors. It was found that the rate of potassium outflow was greatly increased in the presence of NaF or IA, as well as of IA + purine nucleosides. On the other hand, sodium ion transport was practically not influenced by histamine. The effect of histamine on cation transport plotted against time in the presence of 8 mM NaF is shown in Fig. 1. The same correlation in the presence of 1 mM IA + 2.5 mM inosine is demonstrated in Fig. 2.

High rate potassium outflow was accompanied by hydrogen ion uptake which, of course, resulted in a pH shift towards the alkaline side. A highly sensitive detection of this process became possible using the manometric method of Szőnyi (1960). It is seen in Fig. 3 that a significant alkalization, i.e. CO_2 consumption, took place in the presence of NaF. The process was markedly enhanced by

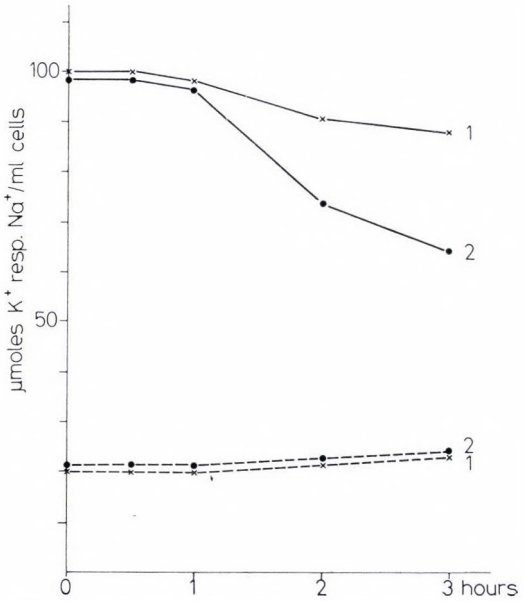

Fig. 2. Effect of histamine on the kinetics of net potassium and sodium ion transport in human erythrocytes in the presence of 1 mM IA + 2.5 mM histamine. For markings cf. Fig. 1

histamine and blocked by EDTA and antihistamines, respectively. The same changes in the pH values of the medium were not observable manometrically in hemolysates, where, of course, no permeability phenomena could occur.

Activation and inhibition of the enhancing effect of histamine on ion permeability

In order to attain a more complete understanding of the mechanism of action of histamine, the effects of a number of substances of different types were tested:

1. It was found that the histamine-effect enhancing permeability was dependent on the presence of free calcium ions in the medium. It is evident from the data of Fig. 4 that with erythrocytes washed with 166 mM NaCl potassium outflow was not increased by histamine in the presence of IA + inosine. However, the effect was promptly manifested, when 2 mM $CaCl_2$ was added to the system. $MgCl_2$ on the other hand was completely ineffective. An experiment carried out with normal (unwashed) blood is shown in Fig. 5. Potassium efflux provoked by histamine was further increased by the addition of $CaCl_2$, while chelating agents, EDTA and EGTA, respectively, completely blocked potassium outflow.

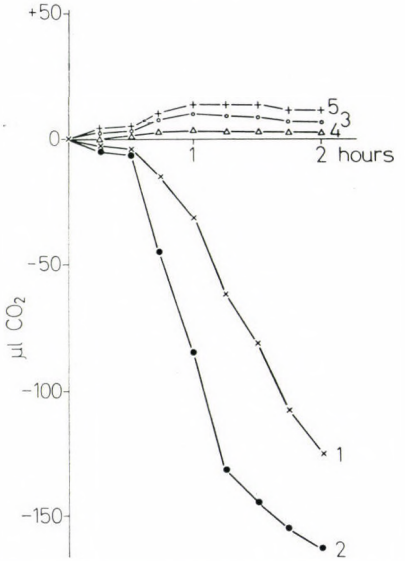

Fig. 3. Kinetics of hydrogen ion uptake in human erythrocytes in the presence of 27 mM NaF. 1: control; 2: 0.5 mM histamine; 3: 1 mM Promethazine; 4: 2 mM EDTA; 5: control vessel containing hemolysate. The Warburg vessels contained 1.0 ml erythrocyte sediment in a total volume of 3 ml with the exception of vessel No. 5 which contained a hemolysate prepared from 1.0 ml erythrocyte sediment by freezing and thawing (twice). The vessels were flushed with a 95 per cent N_2—5 per cent CO_2 gas mixture. NaF was poured into the main vessel after preincubation for 45 min

	K^+ contents at 120 min, µmoles/ml medium
Control	17.9
Histamine	21.0
Promethazine	9.5
EDTA	3.1

2. It has been shown in previous papers (Gárdos, 1956, 1960) that potassium outflow induced by the addition of IA + purine nucleosides is inhibited by arsenate ions. In this case the enzyme nucleoside phosphorylase is unable to synthesize ribose-1-phosphate. Present experiments show that synthesis and metabolic conversion of ribose-1-phosphate is also a prerequisite of the histamine effect increasing permeability. It is seen in Fig. 6 that histamine effect was completely blocked by 5 mM Na_2HAsO_4. On the other hand, the addition of Na_2HAsO_4 at a later time resulted in a considerably lower extent of inhibition. Decrease

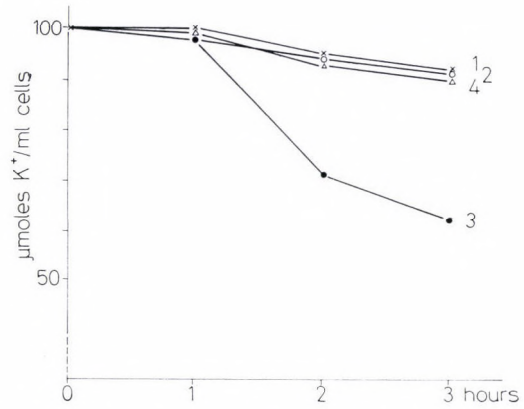

Fig. 4. The role of $CaCl_2$ and $MgCl_2$ in the histamine effect in washed human erythrocytes in the presence of 1 mM IA + 2.5 mM inosine. 1: control; 2: 0.5 mM histamine; 3: 0.5 mM histamine, 2 mM $CaCl_2$; 4: 0.5 mM histamine, 2 mM $MgCl_2$

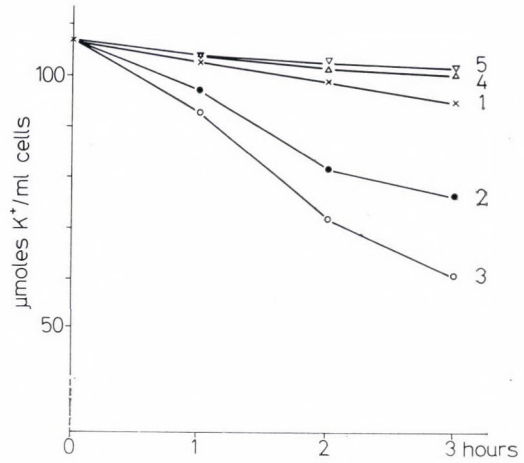

Fig. 5. Effect of $CaCl_2$, EDTA and EGTA on the kinetics of net potassium ion transport in human erythrocytes in the presence of 1 mM IA + 2.5 mM inosine. 1: control; 2: 0.5 mM histamine; 3: 0.5 mM histamine, 2 mM $CaCl_2$; 4: 0.5 mM histamine, 2 mM EDTA; 5: 0.5 mM histamine, 2 mM EGTA

of the inhibitory effect was directly proportional to the degree of the metabolic conversion of ribose-1-phosphate.

3. It is known from recent experiments (Gárdos, 1966a, 1966b) that potassium outflow observed in the presence of IA + purine nucleosides is also inhibited

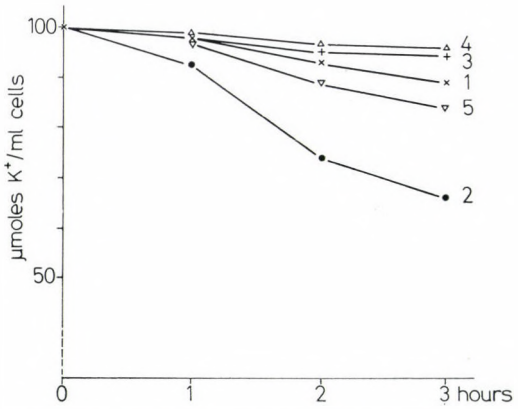

Fig. 6. Effect of $NaHSO_3$ and Na_2HAsO_4 on the kinetics of net potassium ion transport in human erythrocytes in the presence of 1 mM IA + 2.5 mM inosine. 1: control; 2: 0.5 mM histamine; 3: 0.5 mM histamine, 3 mM $NaHSO_3$; 4: 0.5 mM histamine, 5 mM Na_2HAsO_4; 5: blood had been preincubated with 1 mM IA + 2.5 mM inosine + 0.5 mM histamine for 60 min, then Na_2HAsO_4 was added to the system in a final concentration of 5 mM

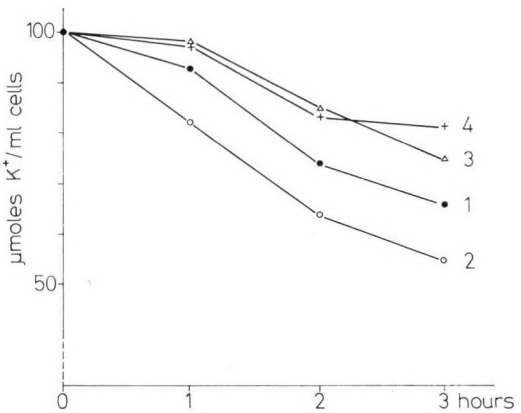

Fig. 7. Effect of nicotinamide, Na-pyruvate and methylene blue on the kinetics of net potassium ion transport in human erythrocytes in the presence of 1 mM IA + 2.5 mM inosine + 0.5 mM histamine. 1: control; 2: 30 mM nicotinamide; 3: 30 mM Na-pyruvate; 4: 0.2 mM methylene blue

by $NaHSO_3$, known to induce the selective breakdown of 2,3-DPG. It is seen in Fig. 6 that $NaHSO_3$ also blocked the permeability promoting effect of histamine.

4. The NAD/NADH system was also necessary for the histamine effect. This was proved by the fact that the efficiency of histamine was increased on

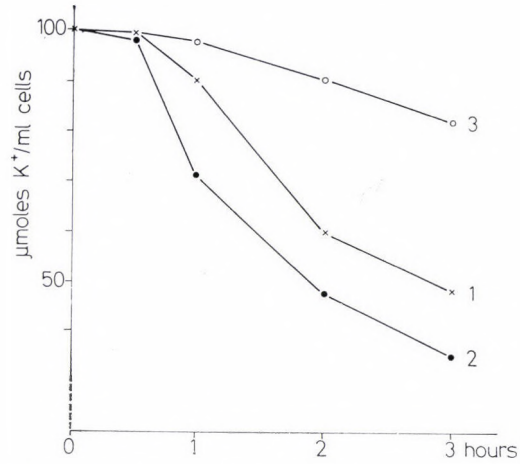

Fig. 8. Effect of nicotinamide and methylene blue on the kinetics of net potassium ion transport in human erythrocytes in the presence of 16 mM NaF. 1: control; 2: 30 mM nicotinamide; 3: 0.2 mM methylene blue

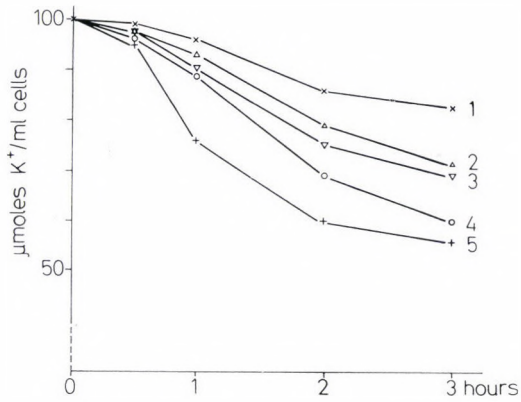

Fig. 9. Effect of NH_4Cl, NaN_3 and imidazoleacetic acid on the kinetics of net potassium ion transport in human erythrocytes in the presence of 1 mM IA + 10 mM inosine. 1: control; 2: 10 mM NH_4Cl; 3: 10 mM NaN_3; 4: 10 mM imidazoleacetic acid; 5: 10 mM NH_4Cl, 10 mM NaN_3, 10 mM imidazoleacetic acid

the addition of nicotinamide. It is known that nicotinamide inhibits the enzymic action of NADase localized in the membranes of the red blood cell (Alivisatos, Denstedt, 1951) and is a precursor of NAD (Preiss, Handler, 1958; Dietrich, Friedland, 1960). Moreover, pyruvate or methylene blue, i.e. compounds which can be reduced by NADH, had an inhibitory effect on potassium efflux brought

about by histamine (Fig. 7). The NAD/NADH system was involved in all cases in which there was a potassium/hydrogen ion exchange. The effect of nicotinamide to activate, and that of methylene blue to inhibit, potassium outflow in the presence of NaF are shown in Fig. 8.

5. In the course of oxidative deamination of histamine imidazoleacetic acid is formed, accompanied by the liberation of NH_3 and H_2O_2 (Tabor, 1951; Mehler et al., 1952; Tabor, Hayaishi, 1955). The presence of these compounds, as well as the addition of NaN_3 (in order to block the action of catalase thus preserving a constant level of H_2O_2) led to an increase in the rate of potassium efflux in the presence of NaF or IA + purine nucleosides or IA + inosine + histamine. Diagrams showing potassium outflow in the presence of IA + inosine are presented in Fig. 9.

6. DNFB inhibited potassium/hydrogen ion exchange in all cases. This is shown in the presence of NaF, NaF + histamine, IA + inosine and IA + inosine + histamine in Table 2.

Table 2

The effect of DNFB on potassium outflow in human erythrocytes

Compounds added	K^+ loss, ΔK^+/120 min μmoles/ml cells	
	without DNFB	with 3mM DNFB
16 mM NaF	19.0	6.0
16 mM NaF, 0.5 mM histamine	34.0	13.0
1 mM IA, 2.5 mM inosine	9.0	6.0
1 mM IA, 2.5 mM inosine, 0.5 mM histamine	19.0	12.0

7. The increased rate of ion transport in red blood cells produced by histamine could be decreased to its original level on the addition of antihistamines. Out of the compounds tested Promethazine (Phenergan, N-dimethylaminopropyl thiodiphenylamine) proved to be the most effective agent in this respect, but Phenindaminetartarate (Pernovin, N-methylphenyltetrahydro-pyridinedene), Tripelenamine (Dehistin, N-α-pyridyl-benzyl-N-dimethylethylenediamine) and Chloropyramine (Suprastin, N-dimethylaminoethyl-N-p-chlorobenzyl-α-aminopyridine) were also highly active (Fig. 10). It should be mentioned that these compounds not only blocked the increased ion permeability induced by histamine, but were also effective in all cases of potassium/hydrogen ion exchange. Such a permeability lowering effect of antihistamines in the presence of NaF + Na_2HAsO_4 is shown in Fig. 11.

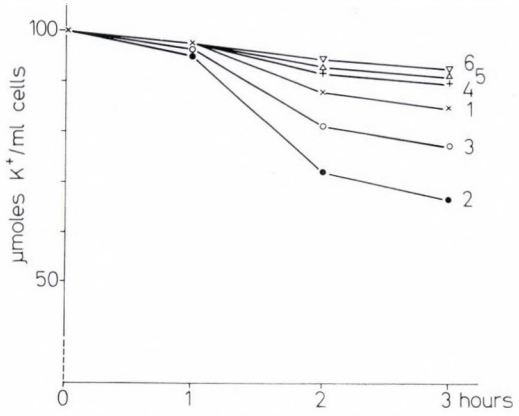

Fig. 10. Effect of antihistamines on the kinetics of net potassium ion transport in washed human erythrocytes in the presence of 1 mM IA + 2.5 mM inosine + 2 mM $CaCl_2$. 1: control; 2: 0.5 mM histamine; 3: 0.5 mM histamine, 1 mM Chloropyramine; 4: 0.5 mM histamine, 1 mM Tripelenamine; 5: 0.5 mM histamine, 1 mM Phenindamine tartarate; 6: 0.5 mM histamine, 1 mM Promethazine

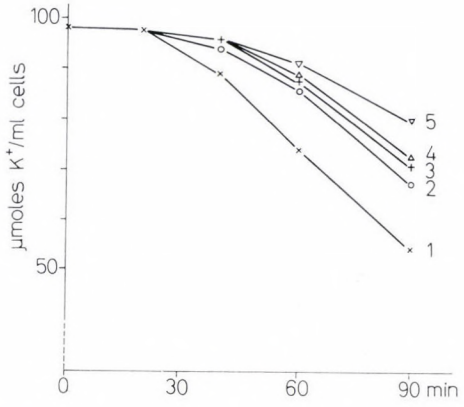

Fig. 11. Effect of antihistamines on the kinetics of net potassium ion transport in human erythrocytes in the presence of 16 mM NaF + 2.5 mM Na_2HAsO_4. 1: control; 2: 1 mM Chloropyramine; 3: 1 mM Tripelenamine; 4: 1 mM Phenindamine tartarate; 5: 1 mM Promethazine

Discussion

The effect of histamine on ion permeability has mainly been studied with the gastric mucosa. Villegas (1963) has shown that passive permeability of potassium ions increased under the effect of histamine. Harris, Frank and Edelman

(1958) have established a direct correlation between potassium and hydrogen ion transport in the gastric mucosa. Cation transport of red blood cells is not influenced by histamine under normal conditions. However, in cells deprived of their ATP content, in the presence of NaF or IA + purine nucleosides the addition of histamine results in an increased permeability to cations. This type of change in permeability is characterized by the fact that only the transport of potassium ions is increased, while sodium transport remains unaffected. The enhanced outflow rate of potassium ions is accompanied by an uptake of hydrogen ions in erythrocytes, leading to a shrinking of the cells, as seen from the decreased hematocrit values (Wilbrandt, 1940; Dunker, Passow, 1950; Szőnyi, 1960). The question arises, whether the mechanism of potassium/hydrogen ion exchange produced artificially in the red blood cells may not be basically identical with that of gastric acid secretion. It is namely very likely that conditions that have been established in red blood cells may serve as a model in the study of the physiological processes of gastric hydrogen ion secretion.

From our earlier and present experimental results the following conclusions can be drawn:

1. In the presence of 8—40 mM NaF or IA + purine nucleosides a high rate potassium outflow (5—20 meq/l erythrocytes/hour) sets in without an equimolar sodium ion uptake. Potassium outflow is accompanied by hydrogen ion uptake.

In our experiments hydrogen ion uptake was demonstrated by a manometric method in the presence of NaF. Manometric measurements of hydrogen ion uptake are not possible in the presence of IA + purine nucleosides because in this case a number of chemical reactions connected with the liberation or consumption of gases are involved:

a. In the presence of IA, ribulose-diphosphate arising from purine nucleosides binds CO_2 with the aid of the enzyme carboxydismutase (Fortier et al., 1967).

b. 3-Phosphoglycerate formed in this way is converted in its turn into lactate. This process results in the liberation of CO_2.

c. Provided that adenosine is used as a purine nucleoside NH_3 produced enzymatically by adenosine deaminase has to be accounted for as well.

2. The rate of potassium/hydrogen ion exchange is increased to a high extent by histamine. The fact that histamine has a maximum activating effect at 0.5 mM concentration and is less effective at concentrations above or below this value suggests that histamine affects cation transport through the activation or possibly inhibition of a certain enzyme. Histamine is known e.g. to increase xanthine oxidase activity (Muraoka, 1962). With regard to the formation of high amounts of hypoxanthine in the course of metabolic breakdown of inosine it is not unlikely that xanthine oxidase activated by histamine, or the electron transfer process coupled with the activity of the enzyme might play a significant role in the increase of ion transport.

3. The experiments presented in this paper point to the fact that potassium/hydrogen ion exchange is a NADH requiring process irrespective of whether the change in permeability is being produced by NaF or by IA + purine nucleosides.

4. The presence of calcium ions is a prerequisite for the enhancement of potassium outflow and of hydrogen ion uptake coupled with it. Calcium forms a link between proteins and phospholipids within the membranes of erythrocytes (Winkler, De Jong, 1941; Bolingbroke, Maizels, 1959). Therefore it is obvious that the lack of calcium leads to structural changes that will completely alter permeability.

5. The mechanism of potassium/hydrogen ion exhange induced in different ways (lead ions, 8—40 mM NaF, IA + purine nucleosides) is probably the same and the compound responsible for the specific change in membrane resistance is in each case most likely histamine. This seems to be supported by the experiments, in which the change in permeability due to either NaF or IA + purine nucleosides was prevented from occurring by antihistamines. Experiments showing that no permeability changes will take place when the NH_2-groups of histamine are bound by DNFB yield further evidence to this concept. On the other hand, if a constant level of histamine is maintained by preventing the oxidative breakdown of histamine (addition of NH_4Cl, NaN_3 or imidazoleacetic acid, respectively), membrane resistance decreases further and the rate of permeability increases.

6. The regulatory role of histamine concerning permeability is manifested only when appropriate metabolic conditions exist. These are provided by the presence of NaF or IA + purine nucleosides. In the presence of arsenate ions the proper metabolic conditions are not brought about anymore by IA + purine nucleosides. The site of action of arsenate is the nucleoside phosphorylase. Therefore ribose-1-phosphate or one of its metabolic products is certainly essential in the induction of permeability changes. It has also been demonstrated in previous experiments (Gárdos, 1967) that whenever IA was not added to the blood at the beginning of the experiments, but only after a 10—40-minute preincubation with purine nucleosides, the rate of potassium outflow was lowered to a considerable extent. These data also point to the fact that the steps which follow the formation of ribose phosphate in the pentose phosphate cycle are of decisive importance in the induction of changes in permeability. In the absence of IA, namely, the oxidative pentose phosphate cycle will operate normally, and glyceraldehyde-3-phosphate and fructose-6-phosphate formed will enter the glycolytic cycle. However, in the presence of IA the enzymes phosphopentokinase and carboxydismutase will catalyze the formation of 3-phosphoglyceric acid from ribose phosphate. 3-Phosphoglyceric acid in its turn will cause a specific decrease of membrane resistance by regulating the rate of the 2,3-DPG shunt. On the other hand, $NaHSO_3$ which is known to cause the selective breakdown of 2,3-DPG will influence metabolic conditions in such a way that no change in permeability can take place.

7. The rate of passive ion movement is certainly determined by the fixed charges of the pores of the cellular membranes. According to Passow (1965) NH_3^+ groups are of paramount importance among the fixed positive charges determining permeability of cells. It is also known that the maximum rate of the downhill transport of potassium and sodium ions respectively, occurs at two different pH values. This suggests that potassium and sodium ions use different channels in their downhill movements (Pfleger et al., 1967). Based on these findings and considerations the role of histamine in increasing the rate of permeability might be explained by its influence on the fixed charges of cell membranes involved in the penetration of potassium.

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Protein Synthesis in the Seminal Vesicle of the Rat

II. The Differential Stimulatory Effect of Testosterone on the Rate of Labeling of Two Protein Fractions

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The rate of incorporation of various radioactive amino acids into the secretory and structural proteins of the rat's seminal vesicles was studied *in vitro*. The secretory proteins were labeled much more intensively than the structural proteins, especially with the amino acids *lysine*, *serine* and *glycine* which have earlier been found at relatively high levels to occur in the basic and/or neutral proteins of the vesicular secretion.

The ratio of the labeling rates of the secretory and structural proteins changed with the *body weight* of the experimental animals: the highest ratio was characteristic of the adult animals. Both immature and very old rats gave lower values. Following castration a significant decrease of the ratio was demonstrated but testosterone treatment of castrated rats completely restored it to the normal value.

The labeling rate of the *secretory proteins* proved to be very sensitive to the actual testosterone supply of the animals. Castration was followed by a rapid decrease and testosterone replacement by a marked (about 50-fold) increase in the rate of labeling of these proteins. Treatment by testosterone of castrated rats resulted in the labeling rate of the *structural proteins* an early 3—4-fold increase, preceding the intensive incorporation of amino acids into the secretory proteins. It was of interest that after castration the labeling rate of the *structural proteins* remained unchanged for at least two weeks.

Introduction

Experimental results in the past ten years yielded ample evidence that one of the early responses to testosterone administration in the male accessory sexual glands is a marked increase in the rate of protein synthesis (Nimni, Bavetta, 1957; Butenandt et al., 1960; Farnsworth, Brown, 1961; Kochakian et al., 1961; Kochakian, 1963, 1964; Costa et al., 1962; Wilson, 1962; Williams-Ashman et al., 1964; Wicks, Villee, 1964; Mosebach et al., 1967; Mányai, 1961, 1962). However, the rate of protein synthesis in these glands has been studied so far merely by determining the rate of incorporation of labeled amino acids into the *total protein material* of these organs. Therefore the main purpose of the present investigations

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was to follow the rate of incorporation of various radioactive amino acids into *different protein fractions* of the seminal vesicles of rats and to demonstrate the effect of castration and of testosterone replacement on the labeling rate of these protein fractions.

The isolation of a protein fraction (APHSE protein: Acid Precipitate Hot Saline Extract) from the rat's seminal vesicles and the assumption of a close relationship between this protein fraction and the proteins secreted by the seminal vesicles have been reported in previous papers from this laboratory (Mányai, Beney, 1961; Mányai, 1964a). Detailed studies on the proteins of the vesicular secretion and further characterization of the APHSE proteins have revealed that the latter represents denatured forms of secretory proteins (or their precursors) which can easily be separated from the structural proteins[1] of the seminal vesicles (Mányai, 1964b; 1965; unpublished 1966; Mányai et al., 1965). In the present experiments tracer studies were made to gain some information about the rate of synthesis of secretory and structural proteins of the seminal vesicles of normal and castrated animals as well as of testosterone treated castrates.

Part of this work has been reported briefly (Tóth, Mányai, 1966).

Materials and Methods

Male rats from the inbred strain of the Institute of Nutrition, Budapest, were used in the experiments. They were fed on normal laboratory diet. The body weights were about 200 g when the stage of sexual maturity was reached. Castration of animals was performed via the scrotal route, under ether anaesthesia. The rats were killed by decapitation, their seminal vesicles were excised and freed from the contaminating secretion. The glands were then pooled in an ice cold Krebs-phosphate buffer solution (Krebs, 1950). The seminal vesicles were finally minced with the help of scissors. The mince was incubated with labeled amino acids.

It has been found earlier (Mányai, 1964a) that the *in vitro* incorporation test is somewhat limited because the rate of incorporation is not linear with time beyond 60–90 min incubation. Consequently we have chosen 30–60 min incubation periods in these studies.

Incubation media (Krebs-phosphate buffer) contained 30 μmoles/ml glucose and 1 to 3 μCi/ml radioactive amino acid. Incubations were carried out in a 95 per cent O_2 and 5 per cent CO_2 atmosphere, under continuous shaking at 37 °C.

The APHSE protein (which contains the proteins secreted by the gland) and the "residual" protein fraction (consisting in bulk of structural proteins) were isolated as described in detail in a previous paper (Mányai, 1964a).

[1] The term: structural protein covers throughout this paper all the proteins of the seminal vesicles except those which are secreted by the organ.

A short description of this procedure is the following: The seminal vesicles are homogenized in ice cold 0.5 N perchloric acid. After the removal of acid-soluble components and lipids the precipitate is extracted by hot 10 per cent sodium chloride solution (APHSE). Nucleic acids and proteins of this extract can be precipitated by ethanol, and subsequently redissolved and reprecipitated. The insoluble residue of the hot sodium chloride extraction is washed by hot and cold perchloric acid and extracted by ethanol and ether, leaving behind the so-called "residual" protein fraction.

The following radiochemical preparations were used:

Compound	Specific radioactivity (mCi per mmole)
[1-^{14}C] glycine	1.125
[2-^{14}C] glycine	1.820
[1-^{14}C] D,L-serine	1.559
[1-^{14}C] D,L-valine	9.890
[^{35}S] D,L-methionine	

all products of the Isotope Institute, Budapest, and

[U-^{14}C] L-lysine	22.800
[U-^{14}C] L-lysine	90.300
[U-^{14}C] L-leucine	30.500
[U-^{14}C] L-proline	68.600

products of the Institute for Research, Production and Utilization of Radio-isotopes, Prague.

Radioactivity determinations were carried out as described in an earlier paper (Mányai, 1964a) with a thin end-window G-M tube. The efficiency of counting was about 7 per cent. Figures calculated for 1 mg protein content were corrected for self absorption whenever the amount of weighed substance exceeded 0.4 mg per cm^2.

Testosterone phenylpropionate (Retandrol) used in these experiments was a product of Richter, Budapest. One mg of the drug was administered in the form of intraperitoneal injection. Control animals were given the vehicle oil only.

The protein content of the APHSE fraction was estimated with the Lowry method (Lowry et al., 1951), horse serum albumin served as standard. The amount of the "residual" protein was weighed directly.

Statistical evaluation of the experimental results was based on Student's "t" test.

Results

First the relative extent of *in vitro* labeling of the APHSE proteins was tested in the mince of the seminal vesicles using various radioactive amino acids. Experimental conditions and results are shown in Table 1. In agreement with earlier data (Mányai, Beney, 1961; Mányai 1964a) the specific radioactivity of the APHSE protein fractions was — although varying according to the amino acid used — 6.6 to 43.7 times higher than the specific activity of the "residual" proteins. The ratios of these specific activities were found with individual amino acids to be comparatively constant in different experiments, and to be independent of the absolute amounts of labeled amino acids added to the incubation media. If the amino acid content (see Table 1) of the basic and the neutral proteins of the secretion (Mányai, 1964b; Mányai et al., 1965) is compared with the determined activity ratios, it is striking to note that lysine, serine and glycine, which occur in high quantities in one or the other secretory protein, are incorporated much more intensively into the APHSE proteins than into the "residual" proteins.

Table 1

Relative rate of incorporation in vitro of various amino acids into the APHSE protein fraction of the seminal vesicles of rats as compared to the levels of the same amino acids in the main secretory proteins

50 mg/ml of the pooled vesicular mince of 4 rats (200 g body weight) and of 10 rats (252 g body weight), respectively, was incubated as described in Methods. Samples were homogenized in ice cold 0.5 N $HClO_4$ (final concentration) and the specific radioactivities of the APHSE protein fraction and of the "residual" protein fraction were determined. In the second experiment the mean values of duplicate incubations are given. Amino acid contents of secretory proteins were calculated on the basis of data published previously (Mányai et al., 1965).

Labeled amino acids		cpm/mg APHSE protein / cpm/mg residual protein	Amino acids μmoles per 100 mg secretory protein	
			basic protein	neutral protein
Exp. 1	valine	15.3	3.84	2.90
	lysine	34.0	8.56	4.51
	glycine	40.2	8.90	10.25
	serine	43.7	6.10	13.60
Exp. 2	leucine	7.2 (6.5; 7.9)	4.35	1.75
	lysine	37.3 (37.8; 36.8)	8.56	4.51
	proline	12.2 (11.4; 13.0)	0.00	2.08
	methionine	6.6 (5.8; 7.4)	1.21	1.00

In the case of other amino acids which are present in the two secretory proteins at relatively lower levels (valine, leucine, proline, methionine) the values of the specific activity ratios are definitely lower.

In the course of further investigations we observed that using the same radioactive amino acid for labeling, the specific activity ratios varied according to the hormonal status of the animals including the degree of sexual maturity and the deprivation or replacement of testosterone.

Changes with the body weight in the relative label of the APHSE protein fraction of the rat's vesicular mince incubated in the presence of [1-^{14}C] valine are shown in Fig. 1.

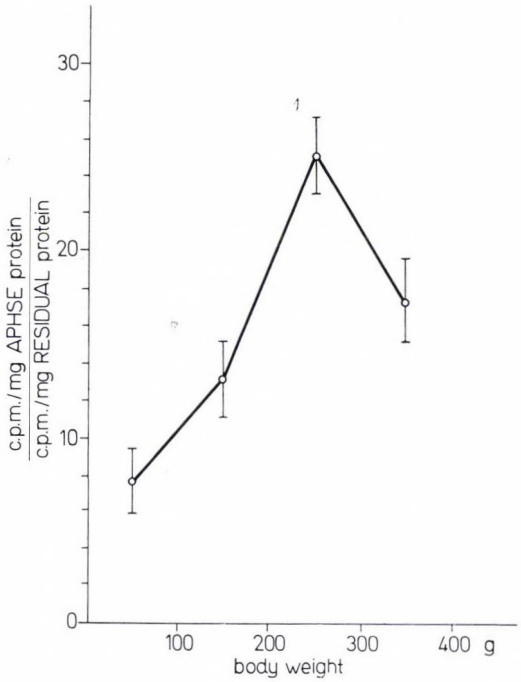

Fig. 1. Incorporation of [1-^{14}C] D,L-valine into proteins of the seminal vesicles of norma rats *in vitro*. The relative labeling of the APHSE protein fraction as a function of body weight of the animals. 100 to 300 mg mince of seminal vesicles of at least 5 normal animals was incubated. Reaction mixtures were then homogenized in ice cold $HClO_4$ (final conc.: 0.5 N). The specific radioactivities of APHSE proteins and of the "residual" proteins were measured as described in Methods. Ordinate: Ratio of specific radioactivities of APHSE proteins and residual proteins. Abscissa: Body weight of animals. Average values of at least ten incubations $\pm$ s.d.

From the analysis of these data it became evident that the high ratios of specific activities were in the first place characteristic of adult animals. The relative rate of [1-^{14}C] valine incorporation was equally lower in young animals and in very old ones.

After castration of normal adult rats the high ratio of specific radioactivities decreased and was restored again to the normal value after administration of testosterone to the castrated rats (Table 2).

Table 2

Relative rate of incorporation in vitro of [1-^{14}C] glycine into the vesicular APHSE proteins of normal, castrated and testosterone treated castrated rats, respectively

The vesicular mince (100 to 300 mg) prepared from the glands of at least 5 animals of 250—300 g body weight each was incubated. The specific radioactivities of the APHSE and the "residual" protein fractions were determined as described in Methods. Mean values of at least twelve incubations per group ± s.d.

Treatment of animals	cpm/mg APHSE protein / cpm/mg residual protein
(A) normal, adult rats	35.8 ± 3.1
(B) adult rats, castrated 14 days before the exp.	5.6 ± 0.6
(C) same as (B) but injected with 1 mg testosterone phenylpropionate daily in the last 6 days before sacrificing	31.3 ± 5.0

Statistical evaluation: A : B $t = 8.9$ $p < 0.01\%$
C : B $t = 5.6$ $p < 0.01\%$
A : C $t = 0.8$ $p = 40\%$

Thus the APHSE protein in the vesicular mince of normal adult rats was 35.8 ± 3.1 times more intensively labeled with [1-^{14}C] glycine *in vitro* than the "residual" proteins of the gland. Two weeks after castration a significant decrease of this ratio to 5.6 ± 0.6 was observed. When castration was followed by testosterone treatment for one week, the ratio increased to 31.3 ± 5.0. From the experiment shown in Fig. 2 it may be seen that this prolonged testosterone treatment increased the incorporation rate of [2-^{14}C] glycine into the "residual" protein by a fa tor of about four. However, the stimulatory effect of testosterone replacement on the labeling rate of the APHSE proteins was much more marked — about fourtyfold — in the experiment.

The above results show that the ratio of incorporation into the two different protein fractions of the castrated animals rises to the normal level after a 6-day treatment with testosterone. It was of interest to investigate the time course of this testosterone effect, by studying the rate of incorporation of the labeled amino acid separately into the two protein fractions.

First the *in vitro* incorporation of [1-^{14}C] valine and [U-^{14}C] lysine into protein fractions of the rat's seminal vesicles was investigated at different time intervals following castration of normal adult rats (Fig. 3). The rate of label-

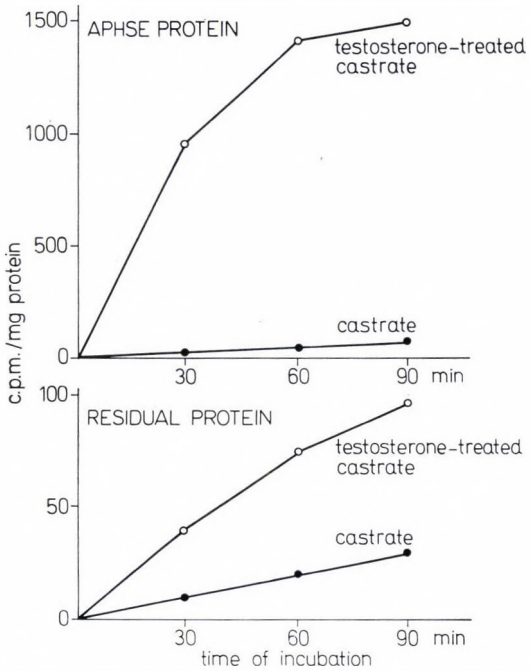

Fig. 2. The differential stimulatory effect of testosterone phenylpropionate treatment on the rate of *in vitro* incorporation of [2-^{14}C] glycine into the two protein fractions of the seminal vesicles of castrated rats. Mince of pooled seminal vesicles of 20 animals (average body weight 280 g) castrated 14 days before, and of 10 animals castrated but treated with testosterone phenylpropionate for the last six days was incubated in 300 mg portions as described in Methods. Reaction mixtures were homogenized in ice cold 0.5 N $HClO_4$ (final conc.) at time intervals indicated on the abscissa. The specific radioactivites of APHSE proteins and of "residual" proteins were determined and shown on the ordinate

ing of the "residual" proteins did not change in the first 12 days following castration. During the same period the rate of labeling of the APHSE protein fraction decreased to a considerable extent: on the twelfth day after castration it was only 32 and 23 per cent, respectively, compared to the same values for normal animals.

In this experiment the amount of extracted APHSE proteins per gram of wet tissue was on the twelfth day 51 and 50 per cent, respectively, of the values obtained with normal rats. On the other hand, the amount of the "residual" proteins was always about 25 per cent of the weight of wet tissue.

The response to a single injection of testosterone phenylpropionate given at different intervals preceding the sacrificing of castrated animals was also different in the APHSE protein fraction and in the "residual" proteins with respect to protein synthesis *in vitro*.

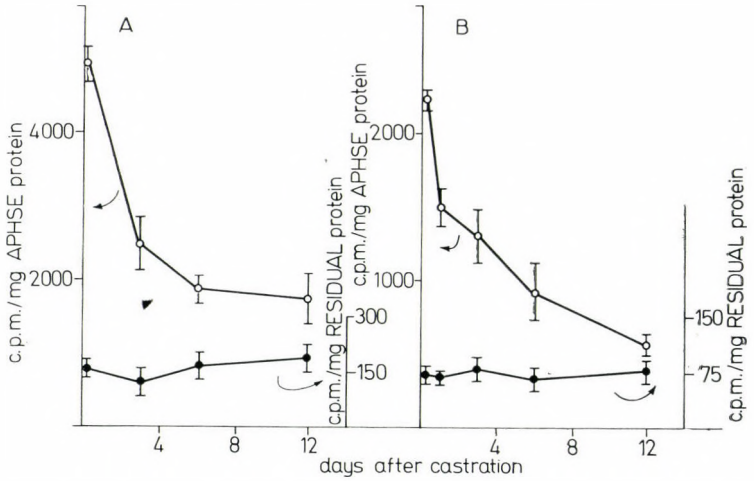

Fig. 3. The *in vitro* incorporation of [1-^{14}C] D,L-valine and [U-^{14}C] L-lysine into the proteins of the seminal vesicles of rats, after different periods following castration. The mince of the seminal vesicles of rats (average weight a: 290 g, b: 180 g) castrated some days before the experiments, as indicated on the abscissa of the diagrams and the mince of the seminal vesicles of intact animals were incubated as described in Methods. The reaction mixtures contained 150 mg of the vesicular mince (prepared from the glands of 4 to 6 animals) and *A*: [1-^{14}C] D,L-valine and *B*: [U-^{14}C] L-lysine. The reaction mixtures were homogenized at the end of the incubation period in ice cold 0.5 N perchloric acid (final conc.), the specific radioactivities of APHSE proteins and of the "residual" proteins were determined and shown on the ordinates of the diagrams. Each point is the mean value of two parallel incubations. Brackets represent the differences of the parallel determinations from the mean values. *Amount of extracted APHSE protein:* normal: *A*: 3.17 $\pm$0.07, *B*: 2.88 $\pm$ 0.07 mg/g wet tissue; on the 12th day: *A*: 1.61 $\pm$ 0.24, *B*: 1.44 $\pm$ 0.14 mg/g wet tissue. *Wet weight of the glands:* normal: *A*: 96.6, *B*: 104 mg/100 g body weight; on the 12th day: *A*: 53.5, *B*: 31.7 mg/100 g body weight

The average values of six experiments of this type are shown in Fig. 4. The amino acid incorporation in the vesicular mince of rats castrated but not treated with testosterone served as a control in each experiment. The effect of testosterone injections is reflected in the relative specific radioactivities of the protein fractions of testosterone treated animals compared to the control ones. As shown in Fig. 4, the specific radioactivity of the "residual" proteins of the gland 18, 45 and 73 hours, following the injection of the hormone was 1.8 $\pm$ 0.3; 2.6 $\pm$ 0.8 and 2.7 $\pm$ $\pm$ 0.4 times higher respectively, than that of the untreated animals. The specific radioactivity (relative to the control) of the APHSE fraction of the androgen treated animals in the 18th hour was very similar (2.7 $\pm$ 0.4) to the value obtained with the "residual" fraction. Later, however, it increased very rapidly and reached a value of 23.7 $\pm$ 3.7 in the 45th hour and 48.3 in the 73rd hour.

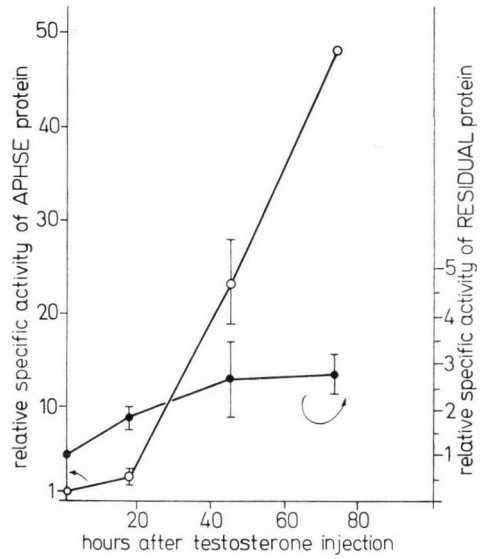

Fig. 4. The *in vitro* incorporation of [1-^{14}C] D,L-valine into the two protein fractions of the seminal vesicles of castrated rats; the effect of testosterone phenylpropionate injections given at different times. Rats (270 g average body weight) castrated 6 to 8 weeks before the beginning of experiments were injected with 1 mg testosterone phenylpropionate at different times before killing, as indicated on the diagram. The reaction mixtures contained 300 mg of the vesicular mince (prepared from the glands of 6 to 15 rats) in the incubating media described in Methods. Following incubation the samples were homogenized in ice cold 0.5 N perchloric acid (final conc.), the specific radioactivities of the APHSE proteins and of the "residual" proteins were determined. The relative specific radioactivities, i.e. the specific radioactivities of these protein fractions relative to the specific radioactivity of the same protein fraction of the control animals are indicated on the ordinate (castrated = 1). Mean values of six experiments $\pm$ s.d. *Amount of extracted APHSE proteins:* control: 7.2 $\pm$ 1.8; 45th hour: 4.3 $\pm$ 1.2 mg/g wet tissue. *Wet weight of the glands:* control: 11.3 and at 45th hour: 23.1 mg/100 g body weight

It was of interest that the APHSE extract of the highly involuted organs of the control animals contained unexpectedly great amounts of protein but almost without any label. These proteins presumably represent proteins with very slow renewal rate, contaminating more or less the secretory proteins extracted from the vesicles of normal adult animals (Mányai, 1964a) and are accumulated relatively in this extract during the involution of the gland. 45 hours after androgen injection the amount of extracted APHSE proteins per gram of wet tissue was about 60 per cent of the control values. At this time the wet weight of the seminal vesicles was doubled. The amount of the "residual" protein was equally about 25 per cent of the weight of wet tissue mince prepared from the seminal vesicles either of control animals or of testosterone treated castrates.

Discussion

The material presented in this paper confirms our previous suggestion (Mányai, 1964a, 1964b; Tóth, Mányai, 1966) that those rapidly labeled proteins which are extracted in denatured form by hot saline solution from a perchloric acid precipitate of seminal vesicles (APHSE) are either identical with the secretory proteins, or represent the precursors of the secretory proteins synthesized in the cells of this organ.

The rate of labeling of this fraction is much more sensitive to castration and to testosterone treatment after castration than the rate of labeling of the bulk ("residual") protein fraction of the vesicles. Testosterone deprivation results in a rapid decrease of the rate of synthesis of the secretory proteins while the rate of synthesis of the structural proteins seems to remain unchanged. It is important to emphasize that this latter observation does not mean necessarily that following castration the rate of synthesis of the structural proteins is not reduced in the vesicular cells. The only conclusion in this respect which can be drawn from our results is that following castration the ratio of the amount of *in vitro* incorporated amino acids to the mass of the proteins is constant in the case of the structural proteins whereas the rate of labeling of the secretory proteins decreases even more rapidly than the amount of the proteins in the APHSE extract.

Testosterone treatment of castrated rats first increases the rate of labeling of the structural ("residual") proteins, the much greater change in the synthesis of the secretory proteins begins only later. Obviously, the high rate of labeling of the APHSE protein fraction (secretory proteins) observed in adult animals and in those which after castration have been treated for some days with testosterone, is one of the ultimate results of the hormone effect. The ratio of the rate of labeling of the APHSE protein fraction to that of the "residual" fractions is a good measure of the hormonal status of the animals. However, the mechanism of the hormone action is not reflected in the rate of labeling of these fractions, and even less in the labeling of the total protein material of the tissue.

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The Induction of a Lytic Enzyme in Cultures of B. Cereus

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The induction of a lytic enzyme in cultures of *B. cereus* 569 was investigated. The production of the enzyme depended on the composition of the culture-medium: enzyme levels of cultures grown on casamine or yeast peptone were significantly higher compared to those of cultures grown in synthetic media. The optimal dose of ultraviolet irradiation with respect to enzyme induction was found to be 7000 to 8000 erg/mm^2. The extent of enzyme formation was higher in young cultures than in older ones.

Enzyme synthesis started 90 to 120 minutes after induction. Final enzyme concentrations in the medium were at least 30 times higher than the original values. A slight accumulation of the enzyme was observed in the cells in the first period, followed by its appearance in the medium as a consequence of the lytic process. Based on results of inhibition experiments carried out in the presence of chloramphenicol it seems to be most likely that the enzyme was synthesized *de novo* in the bacterial cells.

Introduction

Lysis as a consequence of UV irradiation of *B. cereus* 569 cultures and the lytic substance appearing in the medium following the lytic process have been described in previous papers (Csuzi, Krámer, 1962; Csuzi, 1964).

Two different classes of induced enzymes synthesized following phage infection and named early and late enzymes, respectively, have been distinguished. Synthesis and mode of action of one of the proteins of the late period — the lytic enzyme — have been elucidated in some cases. The production of an enzyme resembling in its mode of action lysozyme (it splits glycosidic bonds) has been observed in *E. coli* cells infected by T_2 phage (Koch, Dreyer, 1958; Koch, Weidel, 1961; Weidel, Katz, 1961). The appearance of a lytic enzyme similar in its effect following phage infection of *B. megatherium* has been demonstrated by Murphy (1957, 1960). The lytic enzyme has been found in both cases partly bound to the phage, partly independent from it. The molecular weights of bound and free enzyme were different. Besides these cases lytic substances have been described repeatedly in connection with phage infection of several bacterial cultures.

The lytic enzyme, as one of the enzymes characteristic of the late period, was found to be present in lysogenic bacteria as well. The formation of a lytic

enzyme has been demonstrated in the induction of *E. coli* K 12 λ (Groman, Suzuki, 1963). Enzyme levels after induction were determined quantitatively in the same strain (Jacob, Fuerst, 1958).

Determination of optimal conditions with respect to the formation of the lytic enzyme in *B. cereus* 569 strains, and at the same time a quantitative characterization of the induction of the same enzyme was the purpose of the present experiments.

Materials and Methods

Induction of the lytic enzyme synthesis was investigated on cultures of the *B. cereus* NRRL-569 strain.

Synchronization of B. cereus cultures

Synchronization of cultures was carried out according to the method of Lark and Maaløe by a single shift of temperature (Lark, Maaløe, 1956). Cultures grown at 35 °C were cooled for 20 minutes, when the optical densities of the cultures reached the value of 0.3. After this period the bacteria were warmed to 35 °C in a water bath and were induced by UV irradiation. Number of cells in 1 ml of culture were determined in a Bürker chamber under a phase contrast microscope. The cell number started to increase 20 to 30 minutes after raising the temperature and reached a constant level after another 15 minutes.

Estimation of cell number

A series of dilutions (ranging from a 10-fold to a 10^{10}-fold dilution) was prepared from *B. cereus* cultures. 0.1 ml of each sample of the series was transferred to a casamine medium containing 1.5% agar. The colonies were counted after cultivation overnight at 35 °C.

Determination of intracellular enzyme activity

10 ml samples were taken from *B. cereus* cultures, enzyme synthesis was stopped by the addition of 20 μg per ml of chloramphenicol. The sample was spun and the precipitate was washed with the same (chloramphenicol containing) medium and spun again, finally the cells were mixed with another 10 ml of medium, and disintegrated by ultrasonic treatment in an MSE disintegrator (output: 60 W) for 2 minutes. A solution of the lytic enzyme was treated in the same way and it was proved that the procedure did not influence the activity of the enzyme. Following disintegration insoluble components were removed by centrifugation at 10 000 g. The activity of the supernatant was tested on a *B. cereus* 130 cell wall preparation by the method described in an earlier paper (Csuzi, 1964).

The activity of the lytic enzyme was expressed in arbitrary units. Enzyme samples, one ml of which led to a decrease of the optical density of the cell wall preparation from 0.5 to 0.4 within 20 minutes at 20 °C, were regarded as containing 100 units of enzyme activity.

Protein estimation

Protein containing solutions were dialyzed for 24 hours against water, their protein content was determined by the Lowry method (Lowry et al., 1951). A horse serum albumin preparation crystallized 4 times served as a standard for the calibration of the phenol reagent.

Media

Witte peptone-yeast extract medium was described in a previous paper (Csuzi, Krámer, 1962).

Casamine medium

It differed from the Witte peptone-yeast extract medium insofar that it contained 10 g casamine (Bacto) in one liter instead of yeast extract and peptone.

Synthetic medium

This medium was essentially the same as described by Torriani (1956). It contained the following ingredients in one liter:

NH_4Cl	2.0 g	Sodium citrate	2.6 g
KH_2PO_4	2.7 g	Arginine	0.168 g
$MgSO_4 . 7H_2O$	0.35 g	Glutamic acid	0.320 g
Trace element solution	1.0 ml	Methionine	0.144 g
Iron(II) ammonium		Glycine	0.075 g
sulphate solution	1.0 ml	Tween 80	1.0 ml

Trace element solution

The following amounts of the listed components were present in 100 ml solution:

$ZnSO_4 \cdot 7H_2O$	0.1 mg
$CoCl_2 \cdot 6H_2O$	0.1 mg
$MnCl_2 \cdot 4H_2O$	1.0 mg
$CuSO_4 \cdot 5H_2O$	0.001 mg
$(NH_4)_2MoO_4$	0.1 mg

Iron(II) ammonium sulphate solution

The composition of 100 ml solution was the following:

$(NH_4)_2SO_4 \cdot FeSO_4 \cdot 6H_2O$	0.156 g
Citric acid	0.168 g

Experimental

The first problem to be elucidated was the mode and form of occurrence of the lytic enzyme. Does the enzyme appear bound to bacteriophage particles, or does it exist in a free state after the lysis of cells? In order to decide the question

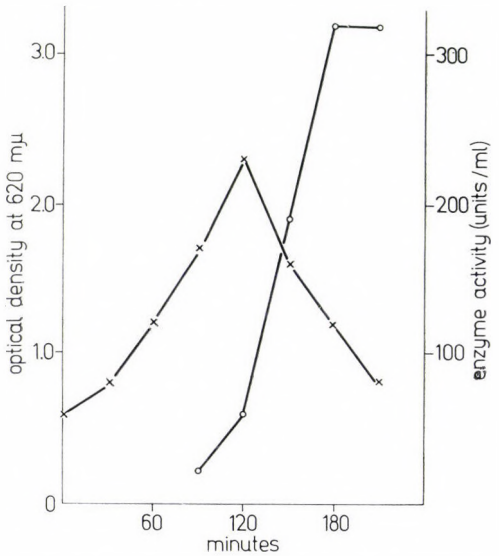

Fig. 1. Variations in the amount of the lytic enzyme in *B. cereus* 569 cultures induced by UV irradiation on casamine medium (dose: 7000 erg/mm²). × — × — × optical density, (dry weights are calculated by multiplying with a factor of 0.25). ○ — ○ — ○ lytic enzyme content in samples of the cultures. Cells were spun at 10 000 g for 10 minutes, enzyme activities of the supernatant were tested on *B. cereus* 130 cell wall preparations

lysates of *B. cereus* 569 were spun at 100 000 g for one hour and enzyme activity of the supernatant was determined thereafter. No loss in enzyme activity was observed, furnishing evidence for the existence of the enzyme in a free state, without being attached to phage particles.

The role of the composition of the medium was investigated in the first place with respect to enzyme induction.

In the early stage of the present experiments lytic enzyme production of *B. cereus* 569 cultures grown on peptone medium completed with yeast was investigated. Optical densities (characterizing dry weight of the cultures) and the increase of lytic enzyme activity following induction by UV irradiation are plotted in Fig. 1. As seen in the diagram the appearance of the enzyme coincided with the lysis of cultures. Optical density of the cultures was reduced to one fourth of its value before lysis. The amount of the enzyme was markedly enhanced after induction. Its original concentration did not exceed 10 units per ml, its highest value was 300 units per ml. Based on these measurements it turned out that at least a thirtyfold increase in enzyme concentration took place in the course of enzyme induction. Rate of growth of *B. cereus* 569 cultures on casamine medium was approximately the same as that on yeast-peptone. Time of lysis following UV irradiation and the increase of the lytic enzyme concentration were identical in both media.

In those cases, where the cultures were grown on synthetic medium, cells were only doubled after UV irradiation and then lysis took place. The concentration of the lytic enzyme — similarly to the conditions discussed above — began to rise 90 minutes after irradiation. The amount of enzyme formed was only 20 to 30 per cent compared to enzyme activities on casamine or yeast-peptone media. This finding refers to the fact that the starting time of lysis was independent of the rate of growth of the cultures. The amount of synthesized enzyme, on the other hand, was proportional to the rate of growth of cultures.

In connection with the assumed inductive effect of UV irradiation it had to be decided whether the effect was a direct one (site of action localized within the cells themselves), or an indirect one exerted on the medium and mediated in this way. In order to rule out the latter possibility the medium was irradiated with UV light, the dose of irradiation varied from 7000 to 14 000 erg/mm^2. Cultures of 0.3 to 0.5 optical density were spun and suspended in the irradiated medium adjusted to the original optical densities. Lysis or formation of the lytic enzyme was not observed on further cultivation of the cultures. On the other hand, if cells of the irradiated culture were separated by centrifuging and suspended in a new medium, the extent of lysis and lytic enzyme formation was the same as in control bacteria.

It has been reported in earlier communications from this laboratory (Csuzi, Krámer, 1962) that the degree of lysis was dependent on the phase of growth of *B. cereus* 569 cultures. The same correlation seemed to be evident with respect to the production of the lytic enzyme as well. As seen in Fig. 2 the increase in optical density of the cultures was accompanied by a decrease in enzyme formation.

Irradiation of bacteria with different doses also influenced enzyme production. The optimal dose with respect to enzyme induction was found between 7000 and 8000 erg/mm^2, higher doses resulted in a constant decrease of enzyme production (Fig. 3).

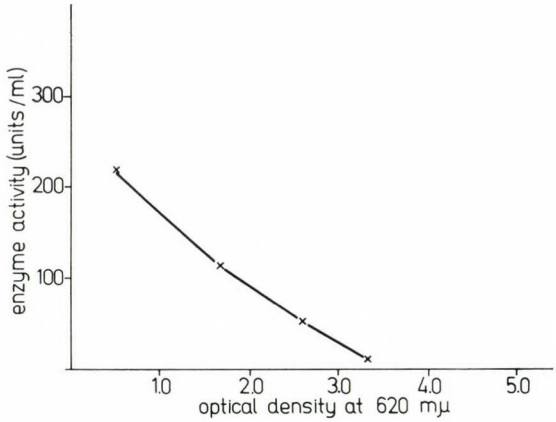

Fig. 2. Lytic enzyme production in *B. cereus* cultures at different phases of growth, induced by UV irradiation (dose: 7000 erg/mm²). Samples were taken from growing cultures at different optical densities. The samples were spun and the cells were diluted with casamine to an optical density value of 0.5 and were irradiated thereafter. After another centrifugation cells were resuspended to the original volume of the samples in casamine medium. Cells were then cultivated for 200 minutes. Following this period the cells were cleaved by an ultrasonic treatment. The amount of the lytic enzyme is plotted as a function of the optical densities of the cultures, determined at the time of induction

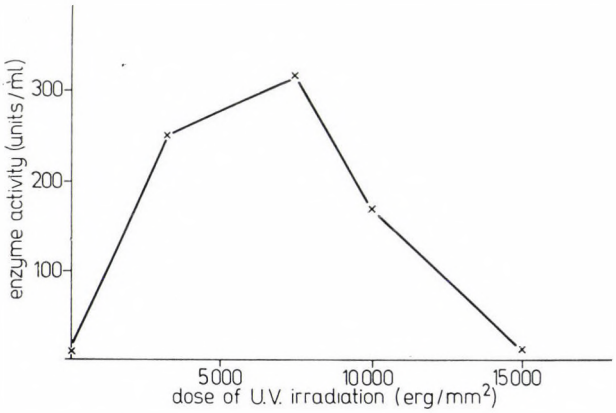

Fig. 3. Lytic enzyme production of *B. cereus* cultures induced by different doses of UV irradiation. 10 ml samples were taken from the cultures 200 minutes after induction. Cells were disintegrated with ultrasonic treatment, insoluble elements were sedimented by centrifuging at 10 000 g and enzyme activity of the supernatant was determined. Abscissa: dose of irradiation in erg/mm² units. Ordinate: lytic enzyme content in the supernatant

It is known that mitomycin C is an effective inductor of episome production (Otsuji et al., 1959; Iijima, 1962). Lysis of *B. cereus* 569 cultures was also inducible by 5 μg per ml mitomycin C. Highest levels of the lytic enzyme were observed

in those cases where cells were treated for 30 minutes with the antibiotic, then centrifuged and resuspended in fresh media and cultured. Growing of the culture, time of the beginning of lysis and the level of the lytic enzyme were the same as those in the control, UV irradiated culture.

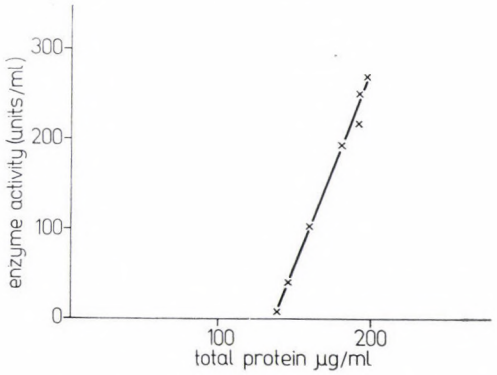

Fig. 4. Lytic enzyme concentrations of *B. cereus* cultures as a function of protein concentration. Ordinate: lytic enzyme content in 1 ml. Cells of the samples taken from cultures were disintegrated by ultrasonic treatment for 2 minutes, and centrifuged. Lytic enzyme activity of the supernatant is indicated on the ordinate. Abscissa: protein in dialyzed supernatant

Quantitative differences in the amount of an enzyme in a given bacterial population may be interpreted in terms of enzyme induction and repression. The amount of synthesized enzyme as a function of the growing of cultures has been characterized by rather simple correlations (Monod et al., 1952).

If the rate of synthesis of the lytic enzyme is plotted against the total cellular protein concentration, a linear correlation may be expected only in those cases, where the majority of cells had been induced previously. Otherwise a rapid increase in protein content of non-induced cells, growing exponentially, may distort the shape of the curves.

By the estimation of the number of surviving cells, data were obtained concerning the proportion of cells which were not affected by irradiation. It was found that the original number of colonies ($8 \cdot 10^7$ per ml) decreased to a value of $3 \cdot 10^3$ per ml following irradiation.

As seen in Fig. 4 the increase in enzyme activity was proportional to the increase of protein content of the culture and remained on the same level, when lysis has finished.

A rapid initial increase followed by a constant value was observed when the ratio of enzyme activity to protein concentration was plotted against time. This is demonstrated in Fig. 5.

Both presentations gave curves, which were very similar to those characteristic of enzyme induction.

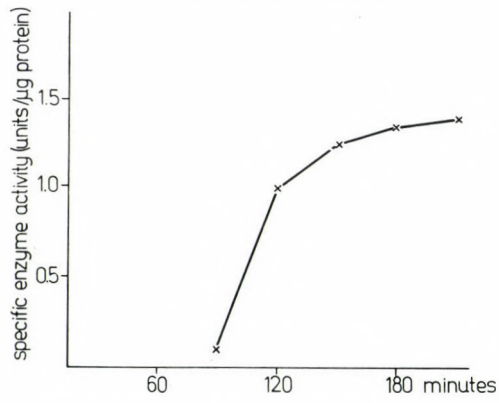

Fig. 5. Ratio of the lytic enzyme and protein concentration of *B. cereus* 569 cultures plotted against time

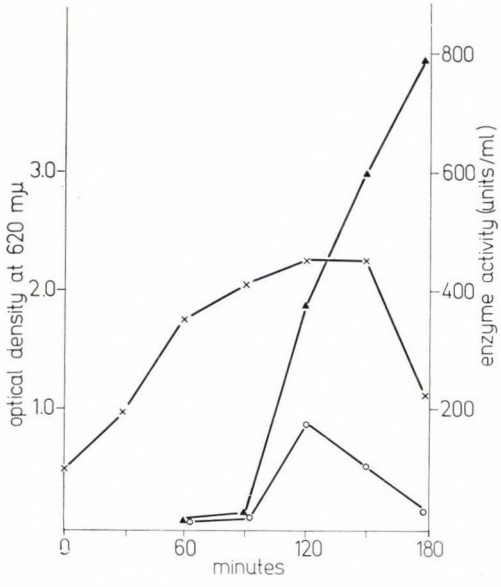

Fig. 6. Variations in lytic enzyme content of *B. cereus* 569 cultures within the cells in the medium. ×—×—× optical density. ○—○—○ intracellular enzyme concentration. 10 ml samples were taken every 30 minutes in order to determine intracellular enzyme activities. Enzyme synthesis was stopped by the addition of 20 µg/ml chloramphenicol. Cells were spun and washed with 10 ml medium containing the same quantity of chloramphenicol. Following another centrifugation step, cells resuspended in 10 ml medium were cleaved by ultrasonic treatment. Enzyme activity of the solution is referred to as intracellular enzyme concentration. ▲—▲—▲ enzyme concentration of the medium. Activities of the supernatants obtained after centrifugation are referred to as enzyme concentrations of media

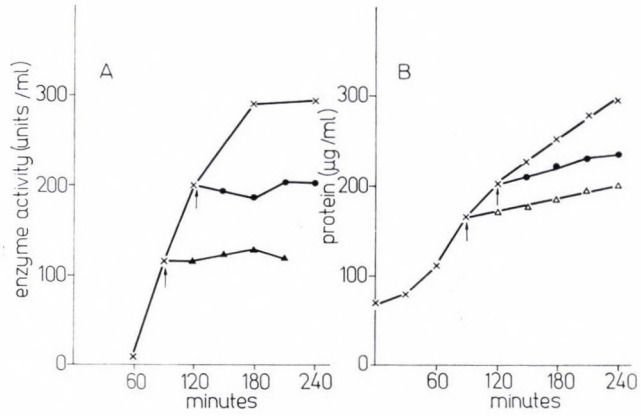

Fig. 7. *a)* Inhibition of lytic enzyme synthesis in *B. cereus* 569 cultures by chloramphenicol. *b)* Inhibition of protein synthesis in *B. cereus* 569 cultures by chloramphenicol. 20 µg chloramphenicol was given to the cultures at the time indicated by the arrow

Lytic enzyme induction in the medium of *B. cereus* cultures was unaltered for 60 to 90 minutes after induction, this period was followed by a rapid increase in enzyme activity starting at the beginning of lysis (Fig. 1). These facts pointed to an accumulation of the enzyme within the cells in the course of synthesis. Following centrifugation and subsequent ultrasonic disintegration of cells, intracellular lytic enzyme concentrations were determined. As demonstrated in Fig. 6 cells were actually enriched in lytic enzyme at the time, when lysis began, later the concentration of the enzyme decreased. It is also seen in Fig. 6 that the enzyme concentrations were much higher in the medium than within the cells. According to the same graph, there has been no detectable enzyme accumulation before lysis. Unsynchronized cell growth might have been responsible for that circumstance. When enzyme synthesis was investigated after synchronizing, intracellular accumulation of the enzyme was observed before lysis. As a further effect of synchronizing, the latency became shorter. Instead of 90 to 120 minutes as determined in unsynchronized cultures, lysis started after 60 to 90 minutes. Intracellular enzyme concentrations were higher as well, but values were still lower than those measured in the medium after lysis.

A further problem to be answered was the following: did the lytic enzyme appear in the cells as a result of a *de novo* synthesis, or did it arise as a consequence of an activating process of a finished protein molecule? Assuming the first case to be true, synthesis should have been blocked by chloramphenicol. It is evident from the data of Fig. 7a that in the presence of 20 µg per ml chloramphenicol enzyme synthesis was blocked completely, while total protein synthesis was not inhibited to the same extent (Fig. 7b).

Synthesis of the lytic enzyme in *B. cereus* cultures started after two generations following irradiation by UV light.

Experiments were carried out in order to clarify whether or not protein synthesis in the course of the latency period was a prerequisite for the synthesis of the lytic enzyme.

Bacterial cultures were incubated for 30 minutes at 37 °C in the presence of 20 μg per ml chloramphenicol given simultaneously at the time of induction. Cells were washed twice with the medium, suspended in fresh medium and cultivated further at 37 °C. Lysis began 90 to 120 minutes after starting cultivation again, concentration of the lytic enzyme in the medium was approximately the same as that of untreated control bacterial cultures.

In another series of experiments, cultures were given 20 μg per ml chloramphenicol, 30 and 60 minutes, respectively, after induction and incubated for another 30 minutes in the presence of the antibiotic. Cells were washed then twice with the medium, and suspended in fresh medium so that the optical densities of the cultures were the same as at the end of chloramphenicol treatment. In cases when chloramphenicol was given 30 minutes after the induction, lysis was observed after another 60 to 80 minutes. In cases when chloramphenicol was given 60 minutes after the induction, another 30 to 50 minutes were necessary until lysis could be observed. Thus the length of the period ranging from the moment of induction until the manifestation of lysis was independent of chloramphenicol treatment.

All these observations suggest that the 90 to 120 minutes latency period and protein synthesis proceeding during this interval was a prerequisite of lytic enzyme synthesis.

Discussion

Induction of the lytic enzyme in *B. cereus* cultures has been investigated in the present work. Lytic activity is not sedimented by centrifuging at 100 000 g, therefore it seems very probable that the bulk of enzyme protein is present in a free state, in the lysates of *B. cereus* cultures and is not attached to phage particles.

The lytic enzyme is inducible by UV irradiation and mitomycin treatment. These inducing factors are known to induce the formation of prophages in lysogenic bacterial strains. The last among the events of inducible protein synthesis in lysogenic bacterial cultures is the synthesis of the lytic enzyme itself, causing finally the disruption of cells. Lytic enzyme synthesis caused by the above-mentioned inducers and simultaneous cell lysis are characteristic of lysogen cultures exclusively. Summing up all these findings, *B. cereus* 569 strains may be regarded as lysogenic ones.

The lytic enzyme, as mentioned already, is not sedimented in the ultracentrifuge, indicating that it is produced as a defective phage protein. One of the manifestations of defective character lies in the lack of association of structural proteins and the lytic enzyme. It is possible that structural proteins are unable to combine with the lytic enzyme, occasionally the protein responsible for this

function may be even absent in the defective phage, on the other hand, molecules of the lytic enzyme may have lost their binding ability as well.

Lytic enzyme content of the bacteria is described — similarly to the enzymes of cellular metabolism — as a function of cellular protein concentration. The increase in enzyme activity is directly proportional to the increase in protein content of cultures. This shows that most cells are induced as a result of UV irradiation. Lytic enzyme formation expressed as a function of the increase in protein content of cultures, variations in specific enzyme activity values plotted against time show correlations resembling those of inducible enzymes in cell metabolism. Furthermore, results of the present experiments are in good agreement with data obtained in a study of T_{6r+} phage infected *E. coli* dCMP (deoxycytidylate) hydroxymethylase formation (Flaks, Cohen, 1959; Flaks et al., 1959). The concentration of this early enzyme of the T_6 phage as a function of cellular protein content varies in the same way as in the case of other inducible metabolic enzymes.

As for the mechanism of lytic enzyme synthesis a definite accumulation of the enzyme takes place within the cells preceding lysis.

A further problem concerning the synthesis of the lytic enzyme is to find satisfactory proof for the *de novo* character of the process. As known from papers of different authors (Nathans, Lipmann, 1961; Rendi, Ochoa, 1962) chloramphenicol inhibits only *de novo* protein synthesis. Complete inhibition of lytic enzyme synthesis has been found in our experiments in the presence of 20 μg chloramphenicol. The same concentration of the antibiotic only partly inhibits cell growth. It has been demonstrated (Sypherd et al., 1962) that synthesis of the inducible enzymes of *E. coli* is inhibited by low concentrations of chloramphenicol, while the formation of other proteins is continued. In this respect the synthesis of the lytic enzyme shows the same extreme sensitivity towards chloramphenicol treatment as the synthesis of the inducible proteins. Our findings in connection with chloramphenicol inhibition furnish considerable evidence for the *de novo* character of lytic enzyme synthesis in the cells of the cultures.

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Biosynthesis of Aldosterone and Other Corticosteroids by Aldosterone Secreting Adrenocortical Tumours in vitro

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Homogenates of adrenocortical adenomas and adjacent cortex from two patients suffering from Conn's syndrome were incubated with Δ^5-pregnenolone-7-α-^{3}H, 11-dehydrocorticosterone-1,2-^{3}H, corticosterone-1,2-^{3}H, progesterone-4-^{14}C and 11-dehydrocorticosterone-4-^{14}C. The biosynthesis of corticosteroids was investigated. After extraction and chromatographic fractionation the individual steroids were identified by radiochemical methods. Aldosterone was synthesized mainly from corticosterone in adjacent cortex and adenomas. Adenoma tissue formed more aldosterone (6 ×) than the adjacent non-tumorous part of the cortex. Tritiated 11-dehydrocorticosterone was converted to 18-OH-11-dehydrocorticosterone and aldosterone. The ratio of cortisol to corticosterone was shifted in favour of the biosynthesis of corticosterone in the adenoma tissue.

Introduction

Adrenocortical adenomas causing "primary aldosteronism" are mostly composed of so-called "hybride cells", having a dense nucleus and pale cytoplasm (Symington, 1960, 1963). Biochemical investigations, including the examination of steroid content (Neher, 1958; Biglieri et al., 1963), perfusion (Schriefers et al., 1963) and incubation studies (Bailey et al., 1960; Davignon et al., 1961; Cavell et al., 1964) with such tumours show that they can form aldosterone, corticosterone and cortisol. Since it is accepted today that aldosterone is produced in the zona glomerulosa (Giroud et al., 1956; Travis, Farrell, 1958; Stachenko, Giroud, 1959; Ayres et al., 1960; Stachenko, Giroud, 1964) and that these cells are lacking the 17-α-hydroxylase enzyme system, the complex biosynthetic behaviour of the adenoma cells required further investigation.

In this work we have incubated tissue samples of adrenocortical adenomas and the adjacent non-tumorous part of cortex from patients with Conn's syndrome and studied the metabolism of progesterone-4-^{14}C, tritiated Δ^5-pregnenolone, corticosterone and 11-dehydrocorticosterone.

Materials and Methods

Adrenal tissues used in this study derived from 2 patients suffering in Conn's syndrome (hypertension, polyuria, polydypsia and hypokalaemia). The urinary excretion of aldosterone (30—35 μg/24 hours) was three times the normal level (6—10 μg/24 hours) in both cases. At surgery yellow coloured adrenocortical adenomas were found and removed.

Case No. 1. The weight of the tumour was 9.8 g. Tumour tissue (450 mg) and adjacent non-tumorous cortex (1500 mg) were homogenized in 4.5 ml and 15 ml of Krebs—Ringer-phosphate solution, pH 7.3, containing 200 mg% glucose and 10^{-3}M Na-fumarate. Samples of the adenoma and adrenocortical tissue homogenates were separately incubated (1.5 ml and 5 ml) with pregnenolone--7-^{3}H, corticosterone-1,2-^{3}H and 11-dehydrocorticosterone-1,2-^{3}H. Details of the incubations are given in the appropriate tables.

Case No.2. The weight of the tumour was 1.63 g. Tumour tissue (530 mg) was homogenized in 8 ml of Krebs—Ringer-phosphate solution, pH 7.4, containing the following cofactors: glucose 200 mg%; glucose-6-phosphate-Na 10^{-3}M; NAD and NADP 10^{-3}M; Na-fumarate 10^{-3}M.

Two separate incubations were carried out with progesterone-4-^{14}C and 11-dehydrocorticosterone-4-^{14}C as substrates. Four ml of the tissue homogenate were used for each of the incubation flasks.

Labelled steroids used in this study were purchased from The Radiochemical Centre, Amersham (Δ^5-pregnenolone-7-α-^{3}H, progesterone-4-^{14}C) and New England Nuclear Corp., Boston, USA (corticosterone-1,2-^{3}H, 11-dehydrocorticosterone-1,2-^{3}H). 11-Dehydrocorticosterone-4-^{14}C was prepared biosynthetically from progesterone-4-^{14}C (Fazekas, Kókai, 1967). The radioactive steroid substrates were dissolved in 0.1 ml of propylene-glycol prior to incubation.

After incubation the steroids were extracted by first adding 2 volumes of acetone to the incubation medium. The aqueous acetone was next filtered and distilled under reduced pressure and the resultant aqueous phase extracted 3 *x* with methylene chloride. The methylene chloride fractions were pooled, dried over anhydrous sodium sulphate and evaporated to dryness. The dry residue was partitioned between 2 × 10 ml of 70% aqueous ethanol and 10 ml *n*-hexane.

The ethanol layers were pooled, evaporated to dryness and the residue was used for chromatographic analysis.

Extracts of incubates deriving from the first case were further analysed by paper chromatography. Separation of steroids was performed on 1 inch wide strips of Whatman No. 1 paper with the descending method by using the following solvent systems: B_1, B_3, B_5 and A systems of Bush (1952), AeB (water : methanol : : benzene : ethylacetate, 300 : 200 : 400 : 100 v/v), a Bush-type solvent system for the separation of 18-OH-corticosterone and the benzene-formamide system of Zaffaroni et al. (1950) running for 24 hours. With each chromatogram reference steroids were run parallel and the R_f values measured. The chromatograms

obtained were first run through a radiochromatogram-scanner (Nuclear Chicago, Actigraph II.) and the radioactive spots were eluted for further identification.

The dried residues of extracts deriving from the incubates of the second case were subjected to purification and preliminary fractionation on 1.5 g silica gel (Davison, grade 12) columns. Solvent system was benzene : chloroform (chl) as follows:

Fraction No.	Solvent, % chloroform in benzene	Volume, ml	Steroids separated
1	5	10	I. Residual progesterone and apolar contaminants
2	10	10	
3	15	10	
4	20	10	
5	25	10	11-deoxycorticosterone
6	30	10	11-dehydrocorticosterone
7	35	10	II. Corticosterone
8	40	10	17-hydroxy-11-deoxycorticosterone
9	45	10	
10	50	10	
11	55	10	
12	60	10	
13	65	10	
14	70	10	
15	75	10	Cortisol
16	80	10	III. Aldosterone
17	85	10	18-hydroxy-11-dehydrocorticosterone
18	90	10	
19	100	10	
20	ethyl acetate	10	

Column fractions were combined in three groups (I, II and III) as indicated and evaporated to dryness.

Fraction I was discarded and steroids were isolated from fractions II and III by thin layer chromatography (TLC). Glass plates coated with a 0.25 mm thick layer of silica gel (Silica gel G, Merck) were used without activation. Prior to use, the silica gel was washed with methanol. The following TLC-systems were used:

TLC-A: chloroform : methanol : water (188 : 12 : 1) (Bennet, Heftman, 1962)

TLC–1: benzene : ethyl acetate (1 : 10) (Raman et al., 1964)
TLC–2: cyclohexane : ethyl acetate (1 : 10)
TLC–4: methanol : ethyl acetate (1 : 20)
TLC–5: chloroform : acetone (95 : 5)
TLC–6: cyclohexane : ethyl acetate (50 : 50)

TLC–7: dichloromethane : acetone (70 : 30)
TLC–8: cyclohexane : ethyl acetate (30 : 70)
TLC–9: dichloromethane : acetone (95 : 5)

Steroids were detected on TLC plates by exposure to I_2 vapours for a few seconds (Matthews et al., 1962). Extraction was performed with methanol by using a zone extractor.

The quantity of steroids was determined by measuring O.D. at 240 mμ against the extract of a blank spot on the same chromatoplate or a paper blank in the case of paper chromatography. The purity of the samples was controlled by readings taken at 225 and 255 mμ.

Steroids were isolated by the addition of non-radioactive carriers. Purification was continued until the specific activity after at least 2 consecutive derivatives and chromatographies differed by less than 10%.

Table 1
Metabolism of pregnenolone-7-α-^3H in human adrenocortical tissue

Compound isolated	μg carrier	Derivative formed	Chromatographic system	μg	SA cpm/μg	Cpm incorporated/g tissue
Progesterone	100	Free steroid	Bush A	70	854	174 580
		Free steroid, no change after CrO_3 oxidation	Bush A	58	872	
11-β-OH-Progesterone	100	Free steroid not acetylable	Bush A	80	105	22 600
		11-keto-progesterone	Bush A	62	113	
Corticosterone	100	Free steroid	Bush B_5	—	—	36 400
		21-acetate	Bush B_1	85	178	
		hydrolysis of the acetate	Bush B_3	64	182	
Cortisol	100	Free steroid	Bush B_5	—	—	31 400
		21-acetate	Bush B_1	75	149	
		hydrolysis of the acetate	Bush B_5	43	157	
Aldosterone	100	Free steroid	Bush B_5	—	—	4 600
		Free steroid	Formamide: benzene	—	—	
		18-21-diacetate	Bush B_3	72	135	
		18-lactone-21-acetate	Bush B_1	44	21	
		18-lactone-21-acetate	Bush B_3	36	23	

5 ml, 100 mg/ml adrenal homogenate in Krebs–Ringer-phosphate solution, pH 7.3,containing 200 mg% glucose and 10^{-3} M Na-fumarate incubated at 37 °C for 2 hours in an atmosphere of 95% O_2 + 5% CO_2

Substrate: Pregnenolone-7-α-^3H, 521 μg, 50 μC.

The quantitative measurement of the radioactivity of ^{14}C-samples was performed on alumina planchettes at infinite thinness with a thin end window G.M. tube at 6% efficiency. The measurement of tritiated samples was performed on copper plates, in a Tracerlab, windowless flow counter using a gas mixture of 98.7% helium and 1.3% butane, at 17% efficiency. The statistical error of counting was less than 5%.

Acetylations were carried out by heating at 60 °C on a water bath for an hour in a mixture of 0.1 ml of pyridine and 0.4 ml of acetic anhydride. Chromic acid oxidation of aldosterone diacetate was performed according to Mattox and Mason (1956). The hydrolysis of steroid acetates was carried out by using methanolic $KHCO_3$ under N_2 (Meyer, 1953).

Table 2

Metabolism of Pregnenolone-7α-^3H in Conn's adenoma tissue

Compound isolated	μg carrier	Derivative formed	Chromatographic system	μg	SA cpm/μg	Cpm incorporated/g tissue
Progesterone	100	Free steroid	Bush A	74	623	
		Free steroid no change after CrO_3 oxidation	Bush A	56	674	445 000
11-β-OH-Progesterone	100	Free steroid not acetylable	Bush A	85	59	42 700
		11-ketoprogesterone	Bush A	66	64	
Corticosterone		Free steroid	Bush B_5	—	—	
	100	21-acetate	Bush B_1	81	129	81 400
		hydrolysis of the acetate	Bush B_3	59	140	
Cortisol		Free steroid	Bush B_5	—	—	
	100	21-acetate	Bush B_1	81	23	16 500
		hydrolysis of the acetate	Bush B_5	39	25	
Aldosterone		Free steroid	Bush B_5	—	—	
	100	Free steroid	Formamide: benzene	—	—	
		18,-21-diacetate	Bush B_3	77	80	23 000
		18-lactone-21-acetate	Bush B_1	45	33	
		18-lactone-21-acetate	Bush B_3	32	35	

1.5 ml, 100 mg/ml adenoma homogenate in Krebs—Ringer-phosphate solution, pH 7.3, containing 200 mg% glucose and 10^{-3}M Na-fumarate incubated at 37°C for 2 hours in an atmosphere of 95% O_2 + 5% CO_2

Substrate: Pregnenolone-7-α-^3H, 521 μg, 50 μC.

Results

The results of the first case are summarized in Tables 1—5. In the pregnenolone incubates the more intensive Δ^5-pregnenolone → progesterone conversion in the adenoma tissue is very apparent as compared to the normal part of the cortex. 11-β-OH-progesterone was isolated from both incubates. The possible role of this compound in the biosynthesis of aldosterone was discussed by Brode et al. (1962). One of the important findings is the increased corticosterone and

Table 3

Metabolism of Corticosterone-1,2-^3H in human adrenocortical tissue

Compound isolated	μg carrier	Derivative formed	Chromatographic system	μg	SA cpm/μg	Cpm incorporated/g tissue
18-OH-corticosterone		Free steroid	Bush B_5	—	—	
	100	Free steroid	AeB	—	—	
		21-acetate	Bush B_1	19	276	16 500
		hydrolysis of the acetate	Bush B_5	8	279	
Aldosterone		Free steroid	Bush B_5	—	—	
	100	Free steroid	Formamide: benzene	92	154	13 400
		18—21-diacetate	Bush B_3	70	62	
		18-lactone-21-acetate	Bush B_1	8	67	
18-OH-11-dehydrocorticosterone		Free steroid	Bush B_5	—	—	65 000
	25	Free steroid	Formamide: benzene	—	—	
		21-acetate	Bush B_3	18	133	
		hydrolysis of the acetate	Bush B_5	11	130	
11-dehydrocorticosterone		Free steroid	Bush B_5	—	—	
		Free steroid	Bush B_1	12	133 000	3 200 000
		21-acetate	Bush A	8	130 000	
6-β-OH-11-dehydrocorticosterone		Free steroid	Bush B_5	—	—	50 000
	50	21-acetate (half sample)	Bush B_3	20	251	50 000
	50	CrO_3 oxidation of free steroid	Bush B_5	12	240	
20-β-OH-corticosterone		Free steroid	Bush B_5	—	—	2 800 000

5 ml, 100 mg/ml adrenal homogenate in Krebs—Ringer-phosphate solution, pH 7.3, containing 200 mg% glucose and 10^{-3}M Na-fumarate incubated at 37°C for 2 hours in an atmosphere of 95% O_2 + 5% CO_2

Substrate: corticosterone,-1,2,-^3H, 200 μg, 40 μC.

decreased cortisol production in the adenoma tissue compared to the adjacent part of the cortex. Aldosterone was synthesized in both incubates, significantly greater amount in the adenoma tissue.

From corticosterone nearly 6 times more aldosterone was formed in the adenoma tissue, and the counts incorporated in all steroids isolated show a more intensive biosynthetic capacity of the enzyme systems including those leading to aldosterone.

In the 11-dehydrocorticosterone incubates the conversion to aldosterone was significant. The compound marked as "20-OH corticosterone" was more

Table 4

Metabolism of corticosterone-1,2-^3H, in Conn's adenoma tissue

Compound isolated	μg carrier	Derivative formed	Chromatographic system	μg	SA cpm/μg	Cpm incorporated/g tissue
18-OH-corticosterone		Free steroid	Bush B_5	—	—	
	30	Free steroid	AeB	—	—	
		21-acetate	Bush B_1	18	480	96 600
		hydrolysis of the acetate	Bush B_5	8		
Aldosterone		Free steroid	Bush B_5	—	—	
	100	Free steroid	Formamide: benzene	95	280	
		18,-21-diacetate	Bush B_1	66	130	76 500
		18-lactone-21-acetate	Bush B_1	6	120	
18-OH-11-dehydrocorticosterone		Free steroid	Bush B_5	—	—	
	25	Free steroid	Formamide: benzene	—	—	26 500
		21-acetate	Bush B_3	11	168	
		hydrolysis of the acetate	Bush B_5	6	161	
11-dehydrocorticosterone		Free steroid	Bush B_5	—	—	
		Free steroid	Bush B_1	9	187 000	7 400 000
		21-acetate	Bush A	6	181 000	
6-β-OH-11-dehydrocorticosterone		Free steroid	Bush B_5	—	—	
	50	21-acetate (half sample)	Bush B_3	14	800	530 000
	50	CrO_3 oxidation of free steroid	Bush B_5	8	810	
20-β-OH-corticosterone		Free steroid	Bush B_5	—	—	6 142 000

1.5 ml, 100 mg/ml adenoma homogenate in Krebs—Ringer-phosphate solution, pH 7.3, containing 200 mg% glucose and 10^{-3}M Na-fumarate incubated at 37 °C for 2 hours in an atmosphere of 95% O_2 + 5% CO_2

Substrate: Corticosterone-1,2-^3H, 200 μg, 40 μC.

Table 5

Metabolism of 11-dehydrocorticosterone-1,2-^3H in human adrenocortical tissue and Conn's adenoma

Compound isolated	µg carrier	Derivative formed	Chromatographic system	Adrenal Adenoma tissue				Cpm. incorp./g tissue	
				µg	SA	µg	SA	Adrenal tissue	Adenoma
Aldosterone		Free steroid	Bush B_5	—	—	—	—	9 400	60 700
	100	Free steroid	Form.: Benz.	—	—	—	—		
		18—21-diacetate	Bush B_3	65	45	77	96		
		18-lactone-21-acetate	Bush B_1	21	47	10	92		
18-OH-11-dehydrocorti-costerone		Free steroid	Bush B_5	—	—	—	—		
	50	Free steroid	Form amide: Benzene	—	—	—	—		
		21-acetate	Bush B_3	—	—	34	62	—	19 500
		hydrolysis of the acetate	Bush B_5	—	—	17	59		
6-β-OH-11-dehydrocorti-costerone		Free steroid	Bush B_5	—	—	—	—		
	100	21-acetate	Bush B_3	80	698	71	596	143 500	385 500
		hydrolysis of the acetate	Bush B_5	32	717	14	584		
Compound not acetylable		Free steroid	Bush B_1 Bush B_3	—	—	—	—	390 000	130 000

Adrenal tissue and adenoma. 5 ml, 100 mg/ml adrenal homogenate and 1.5 ml, 100 mg/ml adenoma homogenate in Krebs—Ringer phosphate solution containing 200 mg% glucose and 10^{-3}M Na-fumarate separately incubated at pH 7.3 and 37°C in an atmosphere of 95% O_2 + 5% CO_2 for 2 hours.

Substrate: 11-dehydrocorticosterone-1,2-^3H, 200 µg, 40 µC in both flasks.

Table 6

Case No. 2. Steroids synthesized from 4-^{14}C progesterone and 4-^{14}C-11-dehydrocorticosterone by Conn's adenoma tissue in vitro

Compound isolated	µg carrier	Derivative formed	Chromatographic system	Chromatographic $R_f \times 100$	SA cpm/µg	Cpm incorp./g tissue/3 hours	% of substrate converted
Deoxycorticosterone	200	Free steroid	TLC—9	66	21.6	153 180	17.0
		Acetate	TLC—5	84	20.7		
17-OH-DOC (S)	200	Free steroid	TLC—8	55	61.3		
		Acetate	TLC—5	55	17.6	65 960	7.3
		Δ^4androstene-, 3,17-dione	TLC—6	60	17.8		
Corticosterone	200	Free steroid	TLC—7	64	9.2	73 000	8.0
		Acetate	TLC—5	31	9.6		
11-Dehydrocorticosterone	200	Free steroid	TLC—7	82	21.0		
		Free steroid	TLC—6	13	5.4	21 000	2.3
		Acetate	TLC—6	75	5.7		
Cortisol	200	Free steroid	TLC—7	40	—		
		Free steroid	TLC—A	20	4.2	33 000	3.7
		Acetate	TLC—1	75	4.5		
18-OH-11-dehydrocorticosterone		Free steroid	TLC—A	53	—		
	200	Free steroid	TLC—4	76	0.7		
		21-monoacetate	TLC—1	76	0.6	2 370	0.26
		hydrolysis of the acetate	TLC—1	28	0.6		
Aldosterone	100	Free steroid	TLC—A	76	—		
		Free steroid	TLC—4	50	—		
		18—21-diacetate	TLC—1	78	3.3	6 125	0.72
		18-lactone-21-acetate	TLC—2	76	3.5		
18-OH-11-dehydrocorticosterone (from 4-^{14}C-11-dehydrocorticosterone as substrate)	200	Free steroid	TLC-A	53	—		
		Free steroid	TLC-4	76	0.3	905	0.5
		21-acetate	TLC-1	76	0.3		

2nd case. Steroids synthesized from 4-^{14}C-progesterone and 4-^{14}C-11-dehydrocorticosterone by Conn's adenoma tissue *in vitro*. 4 ml (265 mg) adenoma homogenate in both flasks in Krebs-Ringer-phosphate solution containing the following cofactors: glucose, 200 mg%; glucose-6-phosphate-Na, 10^{-3}M; NAD, NADP, and Na-fumarate 10^{-3}M incubated at pH 7.4 and 37°C for 3 hours in air atmosphere. *Substrates:* Progesterone 4-^{14}C (135 µg, 243 675 cpm) and 11-dehydrocorticosterone-4-^{14}C(65 µg, 49 855 cpm)

polar ($R_f = 0.69$) than corticosterone ($R_f = 0.82$) in the Bush B_5 system. This substance reacted negatively with blue-tetrasolium indicating the presence of a polyhydroxy side chain, absorbed UV-light and gave the sodium fluorescence reaction. The compound was tentatively identified as 20-β-OH-corticosterone. The excretion of this steroid in the urine was shown by Bulaschenko and co-workers (1960) following the ingestion of corticosterone or ACTH injections.

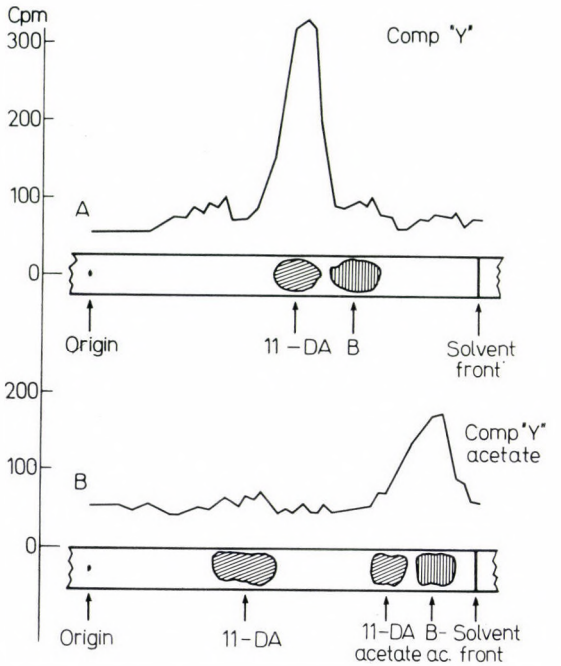

Fig. 1. Radioscannograms of compound "Y" and its acetate. A = Compound "Y" chromatographed in system TLC—7. B = Acetate of Compound "Y" chromatographed in system TLC—1. Abbreviations: 11-DA = 11-dehydroaldosterone, B = corticosterone

The results of the second case can be seen in Table 6. The dry residues obtained by evaporation of fractions II and III of the progesterone-4-^{14}C incubate were dissolved each in 1 ml of methanol and aliquots were used for the isolation and identification of individual steroids. The non-radioactive carriers were added to the aliquots, mixed and run in different TLC systems. Details of the isolation and identification procedures are given in the table. From fraction II, 11-deoxycorticosterone, 17-hydroxy-11-deoxycorticosterone, corticosterone an 11-dehydrocorticosterone were isolated.

From fraction III, aldosterone, 18-hydroxy-11-dehydrocorticosterone and cortisol were isolated. In addition, in fraction III a radioactive substance, desig-

nated "Compound Y" was found, which run parallel with authentic 11-dehydroaldosterone in systems TLC–A, TLC–4, TLC–7. It was more polar than corticosterone. After acetylation the major part of radioactivity separated from 11-dehydroaldosterone in systems TLC–1 and TLC–5 but run with authentic corticosterone-acetate (Fig. 1). This substance was not identified owing to lack of authentic standard but it was synthesized in relatively large amounts, 48 840 cpm/g tissue. After acetylation some residual radioactivity run with the 11-dehydroaldosterone carrier (Fig. 1).

In this incubate 108 690 cpm, 44% of the radioactivity of the progesterone substrate was recovered in the compounds isolated, including "Compound Y".

Column fraction III of the 4-^{14}C-11-dehydrocorticosterone incubate was evaporated to dryness and taken up in 2.0 ml of methanol. 18-hydroxy-11-dehydrocorticosterone was isolated from this incubate. Attempts were made to isolate labelled corticosterone from fraction II but radioactivity was lost from the carrier corticosterone after acetylation. Accordingly, 11-dehydrocorticosterone was not converted to corticosterone in amounts detectable by the method used in this tumour.

Discussion

The conversion of progesterone-4-^{14}C to aldosterone was first demonstrated in perfused calf adrenals by Chen et al. (1958) and in beef adrenal capsule strippings by Travis and Farrell (1958). Raman et al. (1964) described the formation of 18-OH-corticosterone and aldosterone from progesterone-4-^{14}C by mouse adrenals *in vitro*. Mulrow and Cohn (1959) found that human adrenal slices converted corticosterone-4-^{14}C to aldosterone. Based on these results it became apparent that corticosterone is a major biosynthetic precursor to aldosterone. Pasqualini (1964) reported the conversion of tritiated 18-OH-corticosterone to aldosterone in human adrenocortical tissue and adenoma from a patient with Conn's syndrome.

Supported by these findings the biogenesis of aldosterone appears to occur in the following sequence: progesterone ⟶ deoxycorticosterone ⟶ corticosterone ⟶ 18-OH-corticosterone ⟶ aldosterone. This sequence is supported by the present results since from the incubates with added corticosterone-1,2-^{3}H, 18-OH corticosterone and aldosterone were isolated. Moreover the markedly greater amount of counts incorporated into aldosterone from corticosterone compared to the conversion of Δ^5-pregnenolone-7-^{3}H to aldosterone is in favour of corticosterone being first of all involved in aldosterone biosynthesis.

As to the role of 11-β-OH-progesterone that was found in incubates with Conn's adenoma tissue by Brode et al. (1962), and in present experiments, Sandoret al. (1963) using isotope technics obtained evidence for the formation of this compound from pregnenolone and progesterone by duck or chicken

adrenal slices. Kraulis and Birmingham (1964) demonstrated in rat and frog adrenals and Stachenko and Giroud (1964) in beef zona glomerulosa the conversion of 11-β-OH-progesterone to corticosterone and aldosterone at an efficient rate indicating that this compound can be regarded as a precursor to corticosterone, and consequently to aldosterone.

We should like to emphasize the significance of formation of 18-OH-11--dehydrocorticosterone and aldosterone from 11-dehydrocorticosterone. This conversion was previously reported by us (Fazekas, Webb, 1966a, 1966b, 1966c, Fazekas, Kókai, 1967). Since 11-keto-progesterone and 11-dehydrocorticosterone are constantly formed from 11-β-OH-progesterone and corticosterone, these compounds apparently can serve as precursors to aldosterone in normal and especially in pathological conditions.

The quantity of aldosterone per g wet weight of tissue formed in the adenoma incubates was significantly (6×) greater than in the adjacent part of the cortex. This indicates that the adenoma tissue synthesizes aldosterone at a high rate resulting in excess aldosterone secretion with the clinical symptoms of aldosteronism. These results are in good agreement with previous data obtained by the analysis of the aldosterone content of such tissues (Biglieri et al., 1963), or by the *in vitro* incubation of adenomatous and adjacent non-tumorous adrenal tissue without the addition of exogenous steroid substrate (Bailey et al., 1960; Kumagai et al., 1964).

In our experiments the cortisol production was diminished in the adenoma incubations compared to the non-adenomatous part, whereas the synthesis of corticosterone was significantly increased. In the adenoma cells there is apparently a shift in the synthesis of these two important steroids, in favour of corticosterone and consequent aldosterone formation. The same shift can be observed in our second case. The question whether the corticosterone overproduction results in a higher secretion rate was answered by Biglieri et al. (1963), who have found that, although the corticosterone content of Conn's adenomata was increased, the corticosterone secretion rates remained within normal limits in 4 out of 7 patients whereas in 3 cases there was a slight elevation.

Raman et al. (1965) incubated homogenates and subcellular fractions of an adrenal tumour from a patient with primary aldosteronism using progesterone--4-^{14}C, pregnenolone-4-^{14}C and 11-deoxycorticosterone-1,2-^{3}H as substrates. According to their results the preferential pathway for aldosterone biosynthesis was progesterone ⟶ 11-deoxycorticosterone ⟶ corticosterone ⟶ 18-OH-corticosterone ⟶ aldosterone.

The results of our second case show that the tumor tissue synthesized aldosterone, 18-OH-11-dehydrocorticosterone and all the important corticosteroids from progesterone-4-^{14}C *in vitro*. The ratio of 17-α-hydroxyl/17-deoxy steroids formed is 0.39 which is in good agreement with the values found by Brode et al. (1962) in adenomata composed of zona fasciculata type cells, causing Conn's syndrome. Thus these cells have the characteristics of both the glomerulosa

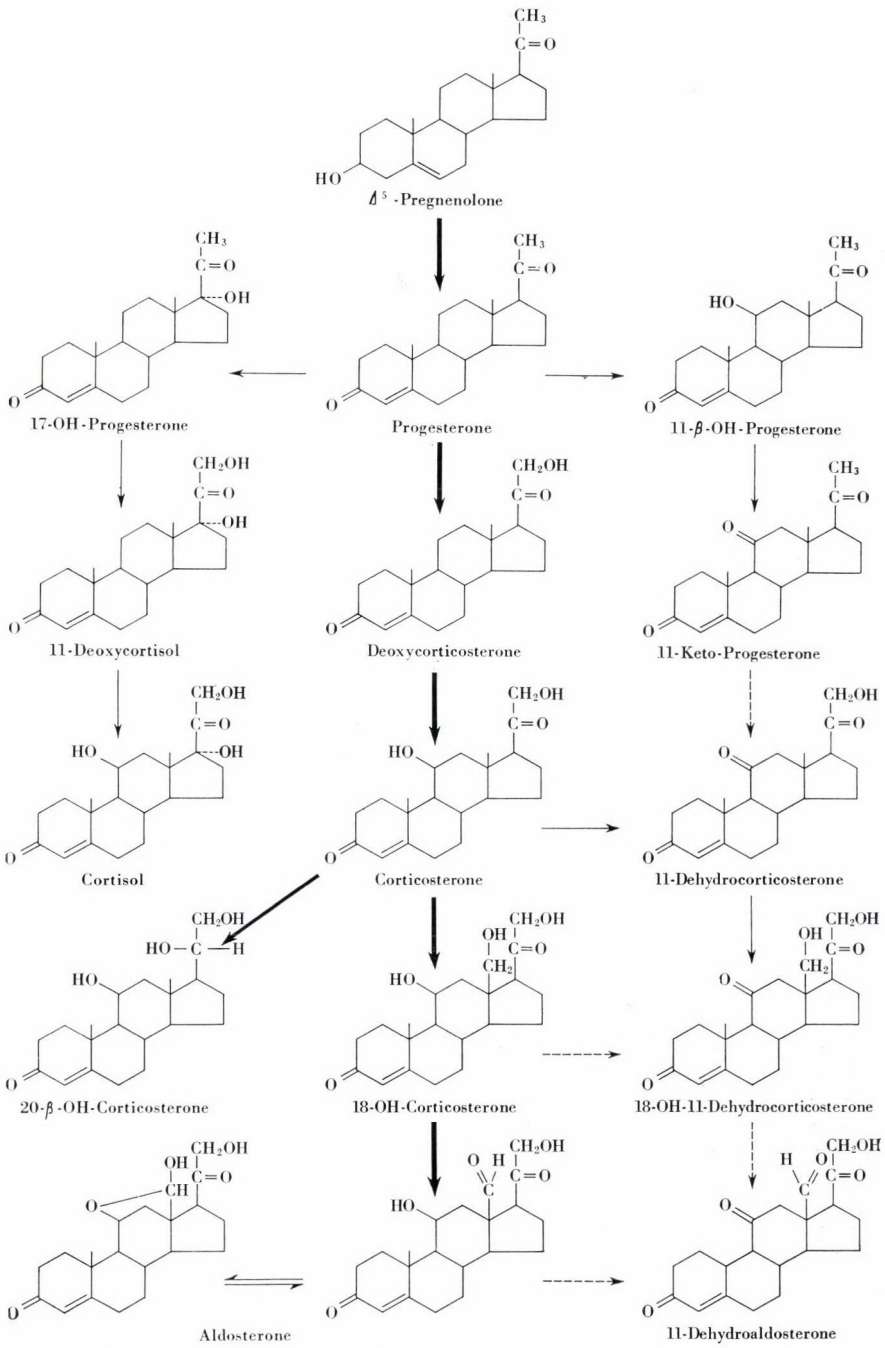

Fig. 2. Main steroid biosynthetic pathways in Conn's adenoma tissue

and fasciculata cells synthesizing aldosterone and cortisol and behaving like a biochemical hybride.

The amount of aldosterone synthesized from progesterone-4-^{14}C was large compared to the value reported by Raman et al. (1965). Nevertheless, we should like to emphasize here the significance of hitherto unidentified metabolites as "Compound Y" in this case and "Compound X" in the case reported by Raman et al. (1965). These substances showed similar properties to aldosterone by chromatography. A substance which might have importance in the pathogenesis of the syndrome is 20-β-hydroxy-corticosterone which was isolated from the incubates of adenomata of primary aldosteronism by Touchstone and Kasparow (1966). This compound was synthesized in large quantities from tritiated corticosterone and tentatively identified by us in the present experiments (case No. 1). These compounds together with the increased production of aldosterone and corticosterone might contribute to the development of the syndrome. Furthermore, 18-hydroxy-11-dehydrocorticosterone was also produced in this tumour from progesterone-4-^{14}C and traces of radioactivity were detected by scanning in the 11-dehydroaldosterone spot (Fig. 1) a compound which is synthesized by the rabbit adrenal (Fazekas, Kókai, 1967a, 1967b).

18-hydroxy-11-dehydrocorticosterone was isolated from the incubate with 11-dehydrocorticosterone-4-^{14}C as substrate in accordance with our previous findings (Fazekas et al., 1966).

In conclusion, the adenoma cells appear to be a biochemical hybride synthesizing large amounts of aldosterone and corticosterone, but also producing various amounts of cortisol, i.e. they have intensive 18-hydroxylase activity and also possess 17-α-hydroxylating enzyme systems with the dominating synthesis of C-21-17-deoxy-corticosteroids.

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Formation and Stabilizing Effect of Disulfide Bridges

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1. The distribution of amino acid residues is very similar in proteins containing and lacking disulfide bridges. The only conspicuous difference is encountered in the frequency of occurrence of basic and free COOH residues. It is suggested that the absence of covalent disulfide bonds are, at least partially, compensated for by the relative excess of the above groups, forming electrostatic and other non-covalent linkages.

2. Three groups of proteins may be distinguished on the basis of actual distribution of disulfide bridges as shown in two-dimensional models. Disulfide bridges may play a decisive role in the stabilization of the conformation of short polypeptides (insulin, trypsin, inhibitor). In comparison, the role of disulfide bridges in the stabilization of larger protein molecules (chymotrypsin, trypsin) appear to be subordinated. Ribonuclease and lysozyme appear to be intermediate between these two groups.

3. The cySS-peptides can be arranged into four, resp. five groups. There is a considerable structural analogy and this suggests a possible phylogenetic relationship.

*

In certain proteins reduction of the disulfide bridges causes inactivation, and under suitable conditions, re-oxidation of the —SH groups results in spontaneous reactivation (Anfinsen, Haber, 1961; Dixon, Wardlaw, 1960; Wilson et al., 1962; White, 1961; Levinthal et al., 1962; Du Yu Chang et al., 1961; Tsou Chen Lu et al., 1963; Meinhofer, Brinkhof, 1963; Du Yu Chang et al., 1965; Nagy, Straub, 1966). From these observations the conclusion has been drawn that all the information necessary for the formation of the native steric structure of globular proteins is contained in their primary structure. The formation of the steric structure of proteins containing disulfide bridges is the result of a sequence of electrostatic and other interactions and "correct" disulfide bridges represent a final state of —SH/SS interchanges (Nagy, Straub, 1966). This implies that the conformation of globular proteins both containing and not containing disulfide bonds are produced by similar forces and disulfide bridges only serve to stabilize an already established thermodynamically stable state.

The primary structure of several polypeptides and proteins has recently been described (Eck et al., 1966). It seemed to be of interest to examine the stabilizing role and the formation of cySS-peptides by a comparison of the structure of proteins which do and do not contain —S—S— bridges.

Table I

Residue distribution in some globular proteins

	Proteins containing disulfide bridges							Proteins not containing disulfide bridges			
Total number of aminoacids (T)	Insulin A + B	Trypsin inhibitor	Ribonuclease	Lysozyme	Trypsinogen	Chymotrypsin	Citochrom C	Hemoglobin α (human)	Haemoglobin β (human)	TMV	T 4 bact. Lysozyme
(T)	51	57	124	129	229	245	104	141	146	158	160
T/basic	12.7	6.3	8.9	7.2	11.4	12.7	4.5	5.9	6.35	11.8	6.15
T/COOH	12.7	14.2	12.4	14.4	22.9	19	8.6	11.7	9.7	11.2	9.4
T/amide	8.5	14.2	7.3	8.0	7.6	9.8	13	28.2	1.63	8.4	8.9
T/aromatic	7.3	7.1	15.5	11.7	13.5	13.6	11.5	12.8	11.2	11.3	12.3
T/hydroxyl	10.2	14.5	5	7.6	5.2	4.8	10	7.05	12.2	4.95	
T/apolar	2.2	2.38	3.45	2.35	2.28	2.0	2.42	1.91	1.8	2.14	2.42
T/polar	1.82	1.73	1.41	1.74	1.94	2.0	1.69	2.16	2.24	1.88	1.76

Distribution of amino acid residues in some globular proteins

In Table I. side chains are classified as apolar and polar, resp., as follows:
Apolar: gly,ala,val,leu,ile,phe,try,pro,
Polar: glu,asp,gin,ain,lys,his,arg,ser,thr,tyr

The frequency of an individual side chain is expressed as the ratio *total number of amino acids/number of the residue.* The smaller is the numerical value, the higher is the frequency of the given residue in the given polypeptide chain.

The distribution of amino acid residues in proteins with known amino acid sequence was compared. Six proteins which contain disulfide bridges and 5 others which do not contain S—S bonds were included in the investigations. The data are presented in Table I.

As shown in Table I there is not much difference in the occurrence of different types of amino acid residues in the two groups of proteins. No significant difference is found if polar, and apolar residues or amide, aromatic and hydroxyl residues are considered.

	Proteins disulfide	Proteins disulfide
Amide	9.23	10.1*
Aromatic	11.45	11.82
Hydroxyl	7.88	8.53

* Hemoglobin is not included in the average.

In contrast to the groups referred to above, considerable differences are observed in the frequency of free-COOH groups and the number of basic residues. In the two kinds of proteins examined:

	Proteins with disulfide bonds	Proteins without disulfide bonds
Basic	9.93	6.92
COOH	15.93	10.10

The frequence of basic and COOH groups is considerably higher in proteins not containing disulfide bridges. In other words, the lack of disulfide bridges in a protein is compensated by a certain excess of groups liable to electrostatic interactions. The latter being, however, weaker than the covalent SS-bridges, their presence will impose a relatively low stability on the proteins against certain denaturating effects.

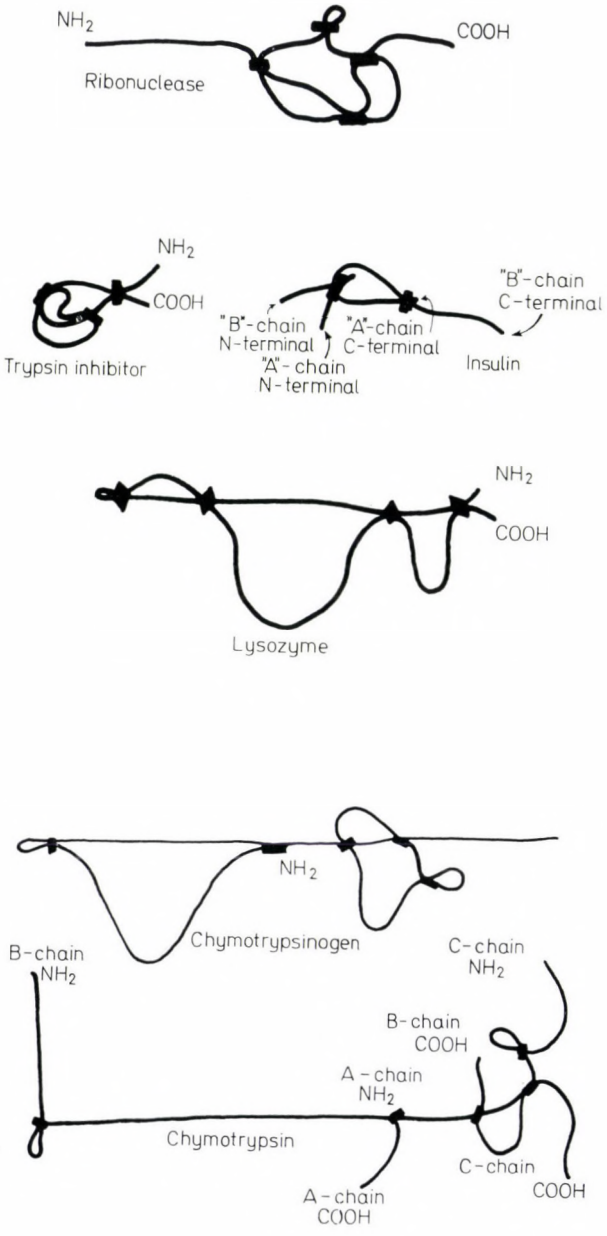
NH2
COOH
Ribonuclease
NH2
COOH
Trypsin inhibitor
"B"-chain
C-terminal
"B"-chain
N-terminal
"A"-chain
C-terminal
"A"-chain
N-terminal
Insulin
NH2
COOH
Lysozyme
NH2
Chymotrypsinogen
B-chain
NH2
C-chain
NH2
B-chain
COOH
A-chain
NH2
Chymotrypsin
C-chain
COOH
A-chain
COOH

Fig. 1

It might seem that all SS-containing proteins hitherto examined are "vulcanized" by disulfide bridges, as suggested by Bresler et al. (1959). However, the descriptive analysis of disulfide bridge containing proteins of known structure does not support the above assumption. The actual distribution of disulfide bridges in the individual molecules are namely different. In certain polypeptides of low molecular weight S—S bonds certainly seem to "vulcanize" the structure, but with increasing chain length this effect appears to decrease considerably.

Two dimensional models of some proteins of known structures containing disulfide bridges were constructed. Each turn of the thread used was considered to represent one amino acid in the chain thus disulfide bridges modelled by a black knot are positioned proportionally. Examination of the simplified two-dimensional models permitted the classification of the molecules under study into three groups. The first group was represented by the short chain insulin, and trypsin inhibitor molecules, respectively, which certainly make the impression of being "vulcanized". Ribonuclease, the representative of group II does not contain SS bridges along a considerable part of its structure (N and C terminal tails). The N-terminal in itself represents 20 per cent of the molecule. Lysozyme also belongs to this group. Group III, represented by chymotrypsinogen and chymotrypsin, in which very small areas are stabilized by disulfide bridges.

Parts of the peptide chains not being stabilized by disulfide bridges appear to contain a relatively large number of polar residues. This is true for a fragment of chain B of chymotrypsin, that between residues number 58—122, i.e. for a section containing 63 amino acids. Chain B contains a total of 11 basic residues and 10 carboxyl groups. Of these, 7 basic residues and 6 acidic residues are present in the fragments 79—87, and 64—78, respectively.

It appears, therefore, that no generalization should be made in respect of the stabilizing effect of disulfide bridges. For any given proteins, the relative number and the actual position of disulfide bridges within the molecule must always be taken into account. Considering the actual distribution of residues, it seems to be highly probable that, with the exception of some polypeptides, covalent bonds do not play a decisive role in either the formation, or the stabilization of the steric structure.

Formation of disulfide bridges

The amino acid sequence in the disulfide peptides of some SS-containing proteins were compared in the following variations:

```
. . . . . A.cys. . . . .                    . . . . .cys.A' . . . .
          |                    and                   |
. . . . . B.cys. . . . .                    . . . . cys.B' . . . . .
```

Table II

Types of disulfide peptides

Ribonuclease	Insulin	Chymotrypsin	Lysozyme	Trypsin inhibitor	Trypsinogen
val.cys ala.cys	val.cys gin.cys	val.cys val.cys H.cys thr.cys	val.cys val.cys arg.cys leu.cys		val.cys val.cys gly.cys ser.cys
ala.cys ala.cys arg.cys gin.cys			gly.cys arg.cys	gly.cys pro.cys	gly.cys ser.cys
tyr.cys asp.cys	tyr.cys val.cys	phe.cys his.cys	tyr.cys pro.cys	phe.cys thr.cys	phe.cys phe.cys his.cys ser. cys
	leu.cys cys.cys	ile.cys ain.cys		leu.cys asp.cys	
		thr.cys ser.cys			thr.cys lys.cys

Table III

Types of disulfide peptides

Ribonuclease	Insulin	Chymotrypsin	Lysozyme	Trypsin inhibitor	Trypsinogen
cys.lys cys.lys cys.tyr cys.tyr		cys.lys cys.lys cys.ala cys.val	cys.lys cys.arg cys.ala cys.glu	cys.lys cys.arg	cys.lys cys.ala
cys.ain cys.arg	cys.ain cys.gly		cys.ain cys.ala	cys.gin cys.met	cys.ain cys.ala
cys.ser cys.glu	cys.ser cys.cys	cys.ser cys.met	cys.ser cys.asp		cys.ser cys.leu
	cys.gly cys.ala	cys.gly cys.gly cys.gly cys.leu		cys.gly cys.leu	cys.gly cys.gly cys.gly cys.leu cys.tyr cys.ala

Structures of the disulfide peptide pairs are given in Tables II and III.

The disulfide peptides of the six investigated proteins could be classified into four or five groups.

As far as the $\begin{matrix} \ldots .A.\ cys \\ | \\ \ldots .B.\ cys \end{matrix}$ variation is concerned, one component of the peptide pair is identical in groups I and V, and analogous in groups II—IV.

I . . . . . val.cys
II . . . . . ala/gly.cys
III . . . . . tyr/phe.cys
IV . . . . . leu/ile.cys
V . . . . . thr.cys

The substitutions observed in groups II—IV were "Standard substitutions" deducible from a single base exchange in the triplet code.

In the peptide pair variations $\begin{matrix} \ldots\ cys \cdot A' \\ | \\ \ldots\ cys \cdot B' \end{matrix}$ shown in Table IV also several analogous peptide pairs were found. These peptides can be arranged into four groups having one component identical or analogous:

I cys.lys/arg
II cys.ain/gin
III cys.ser
IV cys.gly

The substitutions lys/arg and ain/gin are also "standard" ones due to one base substitution in the triplet code.

Certain proteins, being inactivated on reduction and spontaneously reactivated on reoxidation, appear to justify the supposition that all necessary information for the final conformation is contained in the primary structure. Also, it appears highly probable that non-covalent interactions are mainly responsible for the organization of the final conformation in which distant regions of the polypeptide chain take part sometimes. Analogous peptides shown in Tables II and III seem to provide evidence that the information required for the formation of "correct" disulfide-peptide pairs is contained at least partly, in one component of the pair.

It should be noted that no analogies similar to those with cys peptides were observed by comparing the histidine and tyrosine peptides of the proteins studied.

The observed analogies may have several explanations, among others they may reflect certain phylogenetic relationships. Further studies along this line are in progress.

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On the Role of the Z-Lines in the Insect Flight Muscle

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Continuing our previous work concerning the structure and function of the Z-line we treated myofibrils of the bee with a salt solution of high ionic strength in order to remove myosin from its localization. In addition we removed the dense substance of the Z-lines. Even in such myofibrils ATP was able to evoke "contraction band"-like artificial "Z"-lines. We suggest that for the structural changes of the Z-lines no intact A-bands are needed.

In 1956—1959 we performed experiments in which single myofibrils of the flight muscle of the bee were stimulated with microelectrodes (Garamvölgyi, 1959). By strong stimuli we often obtained an irreversible shortening accompanied by a transverse shrinkage at the level of the Z-lines, i.e. by the formation of the well-known "Perlenschnurstruktur" described by earlier authors. In 1958 Ernst interpreted these structural changes with the polyelectrolyte character of the Z-lines (see Ernst, 1963).

Although since that we have no evidence for a similar change in the shape of the myofibril in the course of the physiological contractions, our attention has been directed once more to the possible electrical changes of the Z-line in a quite different way. In connection with microincineration experiments we have observed that the Z-lines of short sarcomeres did not contain inorganic material, while at the sites of the Z-lines of stretched sarcomeres ash substance was present (Garamvölgyi, Kerner, 1962). We attributed this change of the ionic content to structural changes of the Z-lines induced by the change of the sarcomere length.

In the course of the last few years we published a series of papers dealing also with the structural differences of the Z-lines of unstretched sarcomeres on the one hand and of stretched sarcomeres, on the other. We studied this question by utilizing different methods, i.e. interference microscopy (Garamvölgyi et al., 1964), electron microscopy (Garamvölgyi, 1965, 1966) and polarizing microscopy (Garamvölgyi, 1966a, 1967).

In 1965 we observed that in myofibrils deprived of their Z-lines by digestion with lipase, newly formed "contraction band"-like dense lines appeared at the sites of the Z-lines when irrigated with an ATP-containing solution. The appearance of the artificial contraction bands ("Z"-lines) was accompanied by a shortening

of the myofibril (Garamvölgyi, 1965a). On this basis we suggested that the Z-lines were not merely passive constituents of the sarcomere, but could represent perhaps parts of the contractile mechanism. The present paper contains a few additional observations on this subject.

In connection with the eventual functional role of the Z-lines we mention also the paper of Tigyi-Sebes (1966) from our Institute, who observed the A-band substance to migrate towards the Z-lines in the course of the myosin (actomyosin) extraction. In our own paper (Garamvölgyi, Kerner, 1966) we attributed this migration to the binding of the released myosin to the surviving actin filaments.

Methods

The indirect thoracic musculature of 3—4 bees was homogenized in an MSE ultrasonic homogenizer for 15 seconds in a physiological solution ("Pringle's solution"). Drops of this myofibrillar suspension were mounted on slides and observed in a phase contrast microscope. The myofibrils were treated on the slides by applying small drops of the different solutions to the edge of the coverslip. The liquid was sucked through the specimen by a filter-stripe put to the opposite edge.

The following solutions were used for irrigation:

1. Weber—Edsall's solution for the removal of the A-band.

2. A 0.1 per cent solution of lipase enzyme buffered with a 0.067 molar phosphate buffer, pH 7. This solution was used for the removal of the dense material of the Z-lines.

3. A 0.4 per cent solution of ATP, buffered with the same buffer as above, containing also 0.5 M KCl and 0.01 M $MgCl_2$.

Before the start of the experiments Pringle's solution was flowed through the specimen in order to find suitable myofibrils loosely adhering to the slide. Such myofibrils could be expected to remain at the same site during the whole procedure.

Results

To myofibrils of the myofibrillar suspension (Figs 1—3 a) we added Weber—Edsall's solution in order to remove myosin. Figs 1—3 b show the same fibrils as in Figs 1—3 a after the extraction with the salt solution of high ionic strength. The striation pattern is the same as described by Huxley and Hanson for myofibrils deprived of the primary filaments (Hanson, Huxley, 1955; Hanson, 1956; Huxley, Hanson, 1957). The different I-segments are separated from each other by gaps corresponding to the H-zone. In Fig. 3 b the extraction progrediated even further and the I-segment began to be extracted, too. The striation pattern

corresponds to that shown in Fig. 1 e in the paper of Tigyi-Sebes (1966) and to the electronmicrograph shown in Fig. 6 of our own paper (Garamvölgyi, Kerner, 1966). It was necessary to preserve at least traces of the I-segment in order to make possible the observation of the myofibril in the further course of the experiment.

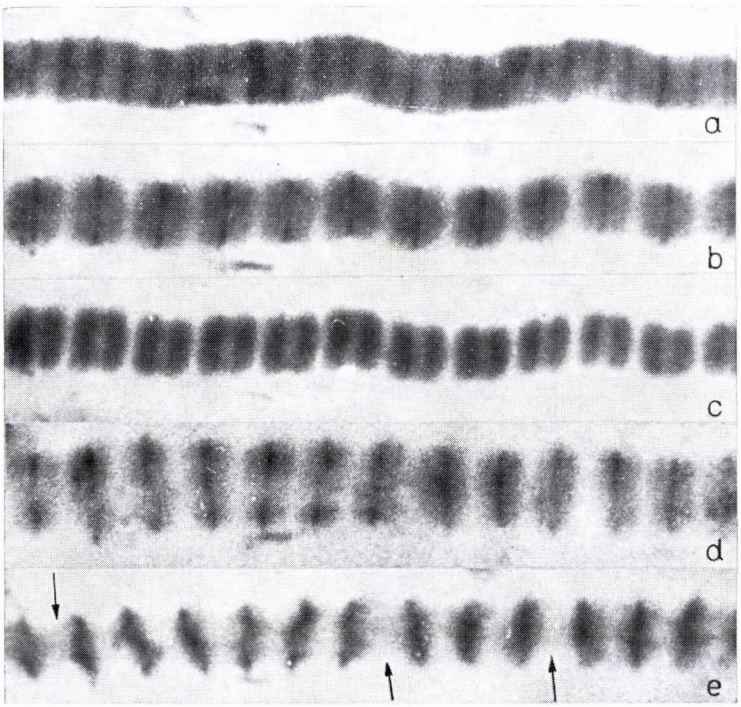

Fig. 1. a: Native myofibril. b: + Weber—Edsall's solution c: +lipase. d: +ATP. e: Additionally washed with Pringles solution. The arrows mark sarcomeres where the stretched S-substance is clearly visible

Subsequently we removed the dense material of the Z-lines by lipase. The result was a light gap in the center of each I-segment (Figs 1—3c). We could only sporadically observe a few traces of the Z-line inside this gap (Fig. 3c, marked with arrows). In general the complete removal of the Z-substance became more difficult after a longer treatment with Weber—Edsall's solution. Simultaneously with the removal of the Z-line the traces of the I-segment became denser.

Pictures d of Figs 1—3 show the state after the addition of ATP. At the sites of the Z-lines artificial "Z"-lines appeared. We could sometimes observe a slight shortening, too, e.g. in Fig. 2 d the wavy myofibril became fairly straight.

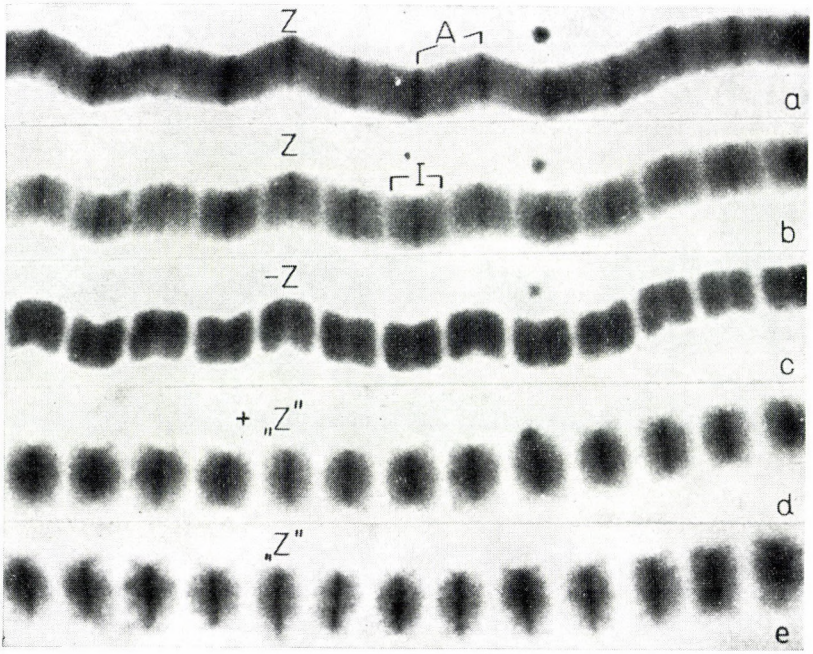

Fig. 2. The same as Fig. 1. The subsequent changes of the Z-lines are indicated at one identical site of the myofibril. In *a*, the length of an A-band,; in *b*, that of an I-segment has been marked. Note the slight shortening in *d*

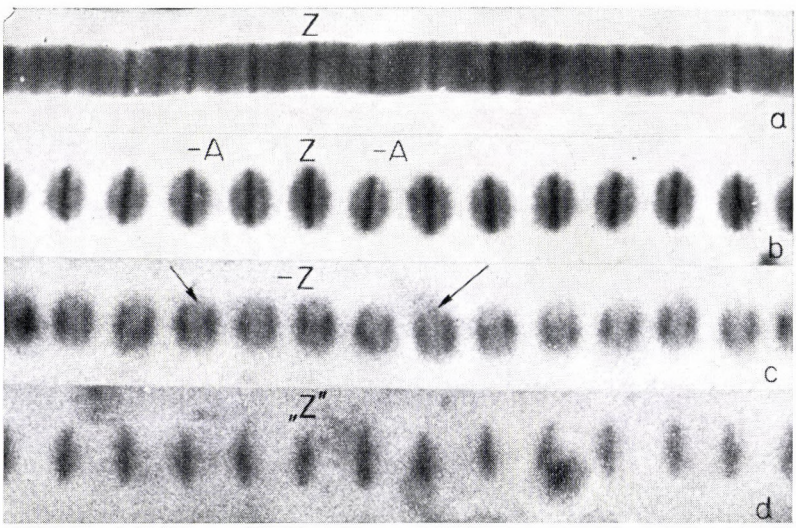

Fig. 3. See explanation of Fig. 1 (the fifth state has been omitted). In *b*, already the extraction of the I-segment began. In *c* some traces of the Z-lines are indicated by arrows

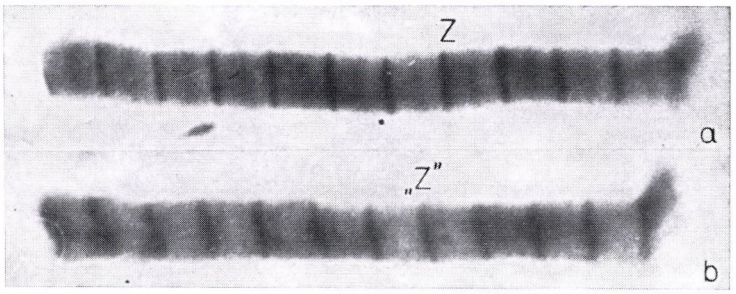

Fig. 4. *a:* Myofibril treated with ATP. *b:* Addition of lipase evoked shortening

By washing the specimen with Pringle's solution the structure became sharper and more contrastful in some cases (Fig. 1e).

In spite of the destruction of the A-band which is indicated by the appearance of the medium gap, the myofibrils never separated into isolated I-segments. This means that inside the H-gap the hypothetical S-filaments (Hanson, Huxley, 1955) in fact represent mechanical connections (Garamvölgyi, 1965, 1967; Garamvölgyi, Kerner, 1966).

In Fig. 4 an other type of similar experiments is shown. To the myofibrillar suspension we added the same volume of ATP-solution and mounted the fibrils on slides in this state (Fig. 4a). Then we added lipase to the specimen and this latter treatment caused shortening (see also Aronson 1963). This is practically the reversal of our former experiments (Garamvölgyi, 1965a) in which first the Z-lines were removed and the shortening induced by ATP followed. Due to the fact that lipase removes the dense Z-material (Guba, 1954; Garamvölgyi, 1965a) we suggest that the shortening of the fibril is closely connected in this case with changes localized inside the Z-lines.

Discussion

A similar result as shown in Fig. 4 has been achieved by Aronson (1963) who used trypsin instead of lipase. He has also come to the conclusion that the enzyme removes the dense substance of the Z-lines. From our own experiments (Garamvölgyi, 1965a) we know that under the influence of ATP the formation of new dense lines takes place. Thus under the simultaneous influence of ATP and lipase the removal of the original Z-material and the accumulation of the "Z"-material might occur in the same time. We can say that the Z-lines visible in Fig. 4a have been — at least partly — transformed into "Z"-lines and this alteration resulted in shortening.

In our experiments demonstrated in Figs 1—3 we had the intention to remove the A-band. It is obvious from the paper of Tigyi-Sebes (1966) that

after the appearance of the light gap in the middle of the A-band the A-band substance is able to leave its original localization. Studying the structure of myosin-extracted myofibrils we have observed that the length of the I-segment is fairly uniform over large areas of the specimen and that the secondary filaments are able to bind one body of the released myosin (Garamvölgyi, Kerner, 1966). On this basis we do not deny that the I-segments, or their traces, in Figs 1—3 may still contain some myosin, but not in the form of separate myosin and actin filaments. We may perhaps say that as a result of the drastic treatments the myofibrillar traces surviving the extraction of both the myosin and the Z-material have not very much in common with the original myofibrillar structure. We are not able to eliminate the possibility that the surviving little amounts of myosin, or actomyosin could contribute in one, or an other way to the formation of the artificial "Z"-lines. On the other hand, it does not seem probable that the structural conditions of a relative sliding movement (Huxley, 1953) could be preserved still. At least no intact A-bands are necessary for the formation of the "Z"-lines.

The single conclusion which we intent to draw from our present experiments is that — in spite of the serious destruction of the myofibrillar structure — the sites of the Z-lines can be still the sites of reactions induced by ATP and may result in the appearance of formations similar to the contraction bands. This fact supports our view that the Z-lines might play an active role in the function of muscle.

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Experiments on the Inward Spread of Cooling of the Brain Surface

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The inward spread of cooling of the brain surface was measured in the experiments performed on dogs. The inward spread of cooling was measured with thermoelectric thermometers and different depths were measured at the same time. The cooling of the brain surface was performed by a cooling thermode operating on the basis of the Peltier effect. Unambiguous conditions of dispersion could be gained only in the state of thermal equilibrium. On the basis of theoretical calculations verified by concrete measurements, a mathematical formula was arrived at according to which by knowing the temperature of the surface of the brain, the temperature of the deeper layers can be determined.

Introduction

General hypothermy has been successfully adopted in neurosurgery because of the decrease of tissue metabolism and accordingly the decrease of the oxygen demand of the brain. Nevertheless, local cooling of the brain is still a problem, with respect to both its indication and application.

After the experimental investigation of the cooling of the brain surface, Negrin (1961, 1964), Ommaya and Baldwin (1962, 1963) have reported its clinical application especially in relation to the treatment of epileptic patients. Negrin (1965) has performed the local cooling of the spinal cord as well. Albin et al. (1965, 1967) have proved the success of local cooling in the case of traumatic injury of the spinal cord in experiments performed on laboratory animals.

In our present study experiments were performed with the brain to investigate the deepness of the cooling effect applied to the surface of the brain, and to determine the temperature of the deeper layers of the brain from the known temperature of the cooled brain surface. Beyond the theoretical aspects this is significant first of all from the point of view of its clinical application, because conclusion can be drawn about the temperature of the deeper layers of the brain without injuring it.

By investigating the inward spread of the cold effect, the following should be considered: First of all the blood supply and blood circulation of the region of the brain to be cooled, the size of the cooling surface, the intensity and measure

of the cooling, the velocity of cooling, the thermal equilibrium formed during the cooling.

The application of a thermode operating on the basis of the Peltier-effect seemed to be the most appropriate for our purposes because by its use defined regions can be cooled under standardized conditions. Stuart et al. (1962) have described thermal electrodes working on the same principle. Some of our results obtained by the same technique have been reported earlier (Pásztor et al., 1965).

Methods

1) *The principle of operation of the cooling thermode and its practical application.* When current flows between two soldered conductors prepared of different materials one of the soldered junctions cools down, according to the Peltier-effect. At the same time the current flow produces heat in the whole circuit (Joule heat). The extent of cooling is determined by the sum of heat loss caused by the Peltier-effect and by the created Joule heat. This is actually the cooling capacity. The maximum of cooling capacity is reached at a certain value of current intensity. By increasing further the current intensity the cooling capacity decreases because the amount of the Joule heat surpasses that of the heat lost due to the Peltier-effect. By simply changing the direction of the current flow the cold and warm polarity is reversed and a well regulated heating can be produced.

The thermode used by us contained "P" and "N" type telluride semiconductor alloys which had a good efficiency in spite of their small size. The optimal current intensity is 12.5 A, which corresponds to 4 W cooling capacity. The cooling thermode used recently has a 7 W cooling capacity at 11 A. When the thermode is in an unloaded state there is 25 to 30 °C difference between the cold and the warm poles. Because the cooling unit carries the heat with drawn from the cold side to the warm one, this side has to be cooled to prevent overheating. This was done by the use of running tap water.

A copper block was fixed on the cooling unit which was in direct contact with the brain surface. An insulated silver spherical electrode was soldered to the bottom of the copper block. This served the purpose of ECoG registration. A bore across the copper block ensured the insertion of a thermistoric thermometer under the center of the cooling region (Fig. 1).

The above arrangement made it possible to cool the brain surface to the desired extent.

2) *Experimental method.* The investigations were performed in two experimental series on 10 and 11 mongrel dogs of different sexes. Narcosis was started with inactin (Na thiobarbiturate) and continued with 100 mg/kg Chloralose or barbiturate given intravenously. The stability of the animal's body temperature was ensured by warming. Extensive craniectomy was performed in the parietal region and after opening the dura the thermode was placed on the brain surface.

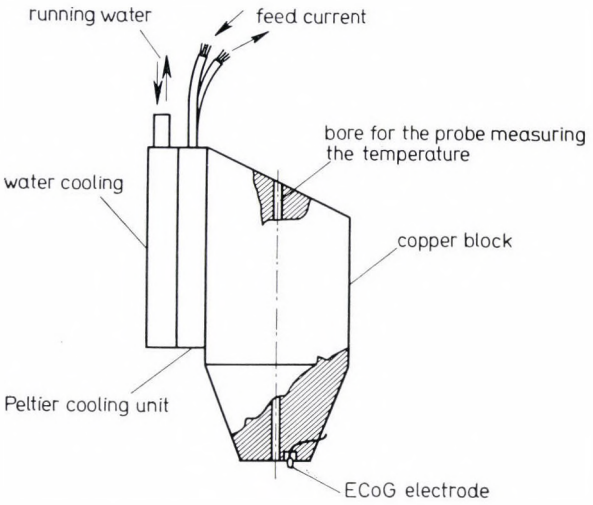

Fig. 1. Sketch of the thermode operating on the basis of the Peltier-effect

In one experimental series the temperature of the cooling brain was measured either on the brain surface or with an inserted copper constantan thermoelectric thermometer. In the other experimental series an insulated probe was inserted in the center of the cooling region through the thermode. The thermistors were fitted on the probe in such a way, that the temperature of the brain could be measured at depths of 5, 2, and 1 mm and at the level of the point of contact of the thermode on the brain surface.

Results

1) *Temperature measured at one point with thermoelectric probe.* The measurements of this experimental series were performed in different depths, and therefore mean values could not be calculated. Fig. 2 demonstrates a typical experiment in which the effect of the cooling of the brain surface was measured by thermoelectric probe inserted 3 mm deep. Rapid cooling was evoked in the experiment and 9 minutes after the cooling started, cooling was stopped before the development of thermal equilibrium. The thermal curve which is not flattened yet in this phase of cooling shows that the thermal equilibrium has not developed yet. Thereafter the brain was allowed to warm up spontaneously for 4.5 minutes and after this the temperature was raised beyond the physiological range. The reason for the 34 °C temperature of the brain is that the thermode placed on the cortex is cooled by running water even if not in operation. In the experiment strychnin-

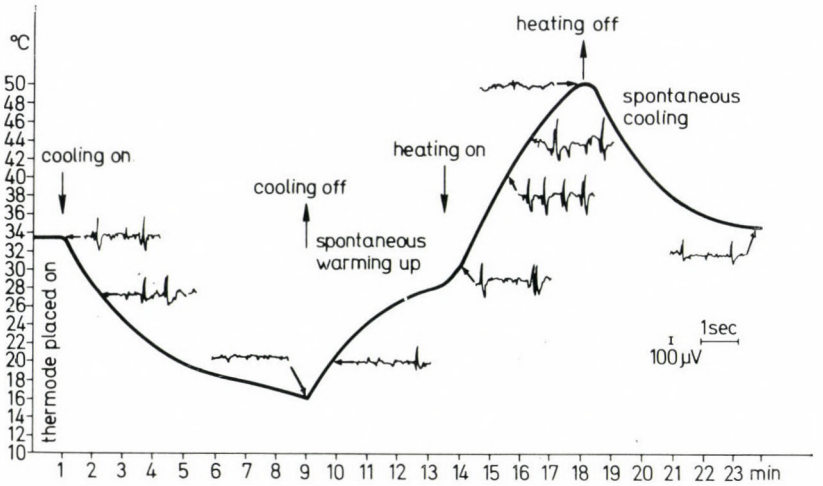

Fig. 2. Change in temperature of the brain due to local brain surface cooling and warming measured at a depth of 3 mm. The changes in strichnin-spike activity brought about by a 0.5 per cent strichnin solution under the thermode are marked beside the adequate temperature. The ECoG registration was performed from the silver spherical electrode built into the bottom of the thermode (angular electrode of steel fastened to the skull above the sinus frontalis served as an indifferent electrode). Voltage calibration and time markings are identical on all registrations

spike activity was evoked by using a 0.5 per cent strychnin solution beneath the thermode. The changes in the activity as a function of temperature are shown in the curve. It should be emphasized that the temperature measured in a depth of 3 mm with respect to the brain surface means significantly lower temperature.

2) *Thermal changes measured in several depths at the same time.* Detailed calculations were made of the data of the precisely localized thermistoric measurements both in the phases of cooling and warming. Altogether 31 coolings and 26 warmings were performed. The cooling was performed with a thermode of 7 W cooling capacity and of great inertia of heat because of the great mass of the copper block. The mean value of the maximal rate of cooling with this thermode was 2.5 °C/min measured at the brain surface and 0.75 °C/min measured in 5 mm depth. The mean value of the temperature reached was 9.5 °C on the brain surface, 11 °C at 1 mm depth, 14.5 °C in 2 mm depth, and 20 °C in 5 mm depth. These latter values refer to a steady state thermal equilibrium. The set in of the steady-state took 30 minutes on the average. The temperature was registered systematically in the course of cooling till the development of this state of equilibrium. After the development of the state of thermal equilibrium cooling was stopped and heating was performed in such way that the physiological value should be reached in about 5 minutes. Under such circumstances the maximal rate of heating was on the average 7 °C as measured on the surface.

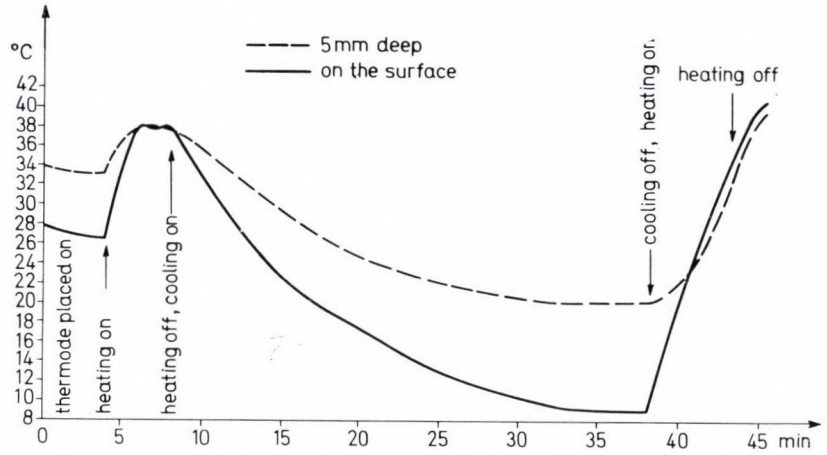

Fig. 3. Summation of the data of 31 local brain surface coolings and 26 warmings performed on 11 dogs with the indication of the temperature values measured on the surface of the brain and at a depth of 5 mm. (Details see in text)

The data of the measurements of this experimental series are shown in Fig. 3

3) *Theoretical calculations.* Irrespective of the data measured we made an effort to determine the distribution of the temperature evoked by cooling on the brain surface. For this purpose a mathematical formula was developed. This formula permits the determination of the temperature of the deeper layers of the brain if the temperature of the brain surface is known. For the calculations the following assumptions were made:

While cooling the brain surface, the heat produced by the cortex is withdrawn by the thermode, and the brain cools down. In steady-state the heat withdrawn by the thermode and the heat produced by the surface layers of the brain are equal and the temperature dispersion becomes constant.

In relation to heat production it was considered that the blood flow of the cortex is 4 to 5 times greater than that of the white matter, and thus the way it produces heat is similar. Therefore the surface of the brain is considered as a layered and equally divided lamellar heat source.

Detailed derivation of the mathematical formula is discussed in the Appendix.

According to the above described, the following equation determines the temperature dispersion of the part of brain beneath the thermode in steady-state in relation to the surface of the grey matter:

$$T = T_f e^{-m_1 X} + (T_H - T_f e^{-m_1 H}) \frac{\mathrm{sh} m_1 X}{\mathrm{sh} m_1 H} \quad \text{if} \quad 0 < X \leq H$$

In relation to the white matter the temperature dispersion is described by the following equation:

$$T = T_H e^{-m_2(X-H)} \text{ if } X > H$$

where

$$T_H = \frac{m_1 T_f e^{-m_1 H}(1 + \text{th} m_1 H)}{m_1 + m_2 \text{th} m_1 H}$$

The symbols used in the equations are the following:

T	$= \vartheta m - \vartheta$	The measure of temperature decrease in °C
T_f	$= \vartheta m - \vartheta_f$	Temperature decrease of the brain surface in °C
T_H	$= \vartheta m - \vartheta_H$	Temperature decrease of the interface in °C
ϑ_H	$=$	Temperature developed at the interface in °C
H		Thickness of the grey matter in mm
X		Distance measured from the brain surface in mm
K		Intensity factor, quotient of the heat source intensities of the grey matter and of the white matter.
q_0		Heat source intensity of the white matter
λ		Heat conduction factor of the brain
m_1, m_2		Auxiliary amounts

The limit of validity of the mathematical formula is that depth in relation to which the area of the cooling surface is relatively large. E.g. in the case of applying a thermode of 30 mm in diameter, the spread of the temperature can be calculated precisely to about 5 mm depth.

Discussion

1) *The efficiency of cooling.* Our data obtained indicate that by the thermode applied by us, effective cooling can be produced to a depth of about 6 to 7 mm. This statement seems to contradict the informations obtained when deeper layer of the brain were cooled. In such cases freezing even to —80 °C was effective for only short distances.

Mark et al. (1961) have measured a temperature as high as 22 °C at a distance of 2 mm upon cooling to —4 °C by means of a freezing probe. Miyazaki et al. (1963) in their experiments performed on animals have measured the temperature of the brain frozen in the region of the subcortical nucleus at distances of 2 mm, 4 mm and 6 mm from the end of the freezing probe. In the case of

quick freezing from 0 °C to —20 °C in 15 seconds they measured +15 °C at a distance of 2 mm and 38 °C at a distance of 6 mm from the point of freezing. In the case of slower cooling (10 minutes) when —20 °C was reached at the end of the freezing probe, the temperature was +5 °C at a distance of 2 mm, 13 °C at a distance of 4 mm and 18° C at a distance of 6 mm. Coe and Ommaya (1964) have performed experiments in which by producing —45 °C at the end of the freezing probe, they reached an average temperature of +10 °C at a distance of 5 mm.

Cooper and Gioino (1965) have performed stereotactic operations on human beings by freezing. During the operations the temperature of the canule applied for 2 minutes was —80 °C, the temperature measured in the subcortical ganglion at a distance of 1.4 mm was —15 °C, and at a distance of 3 mm it was +3 °C.

In our own experiments when below the thermode on the brain surface the temperature measured was 9 °C, even at a depth of 5 mm significant cooling was observed (20 °C).

The relatively smaller cooling effects obtained by the use of a freezing probe can be attributed to several reasons. One essential reason is that the brain also freezes around the freezing probe. Thus much energy is expended on the freezing heat, and besides, the thermal conductivity of the frozen brain also lessens. Owing to the small surface of the freezing probe its cooling capacity is also small. The freezing probe is surrounded from each side by brain tissues (in most cases by the grey matter, supplied with a greater blood flow), which further decreases the cooling capacity. In the investigations cited above no efforts were made to reach thermal equilibrium or, due to the nature of the experiments it was not necessary.

In our own experiments the surface of the thermode was relatively large, and the cooling capacity significant. Our aim was not to bring about irreversible changes in the surface matter of the cortex. Therefore, the temperature was not decreased to the freezing point, and thus the poor thermal conductivity of the frozen brain tissue could not deteriorate the cooling capacity.

This latter assumption is supported by the experiment of Klatzo et al. (1958). They placed a sheet of steel cooled down to —50 °C on the surface of a cat's brain and observed a decrease of temperature to only +27 °C at a depth of 5 mm in 20 sec. In this experiment thermal equilibrium is out of question because the aim of the experiment was not the determination of the temperature, and the cooling lasted for a short time. Greater cooling was doubtlessly prevented by the freezing of the surface of the brain leading to the decrease of its thermal conductivity.

Our experiments prove that the circumstances of the inward spread of the cooling effect can be determined correctly only in the state of thermal equilibrium. On the other hand, the temperature is greatly influenced by the speed of cooling, as shown also by the experiments of Miyazaki et al. (1963).

2) *Mathematical formulation of the distribution of temperature.* Walder (1967) has developed a mathematical formula for the calculation of the diameter of the frozen brain in connection with the stereotactic freezing of the deeper layers of the brain. He has taken into consideration the temperature of the freezing probe (from —10 °C to —180 °C), the diameter of the freezing probe, the heat conduction factor of the ice, and that of the brain. This equation differs from our own mathematical formula not only because it refers to the frozen brain, but also because it describes the spreading of the cooling effect in the deeper homogeneous matter of the brain, as discussed by us.

If our experimental conditions are substituted in the mathematical formula developed by us, the temperature calculated for a given depth is in a good agreement with the actual values measured in the experiments performed on animals.

In addition to the laws of thermodynamics biological factors such as the difference of the heat production between the cortex and the white matter were also considered. At this point the contradictory results obtained by Cooper and Gioino (1965) have to be mentioned. The authors themselves thought it interesting to mention the fact that the measured heat distribution around the freezing probe gave identical values in the white matter and in the subcortical grey nucleus. In this respect we repeat our earlier observations concerning the freezing probe and the undeveloped state of thermal equilibrium. The limited blood flow in the white matter and its decreased heat production have to appear in the spread to higher depths of the cooling effect.

Concerning the application of the mathematical formula it should be stressed that the equation referring to the white matter, can be applied only in that case, when there is a cortical layer, several millimeters thick, above it. At the same time the calculations could not be complicated further the considering that sulcus or some deeper located cortical matter could also be involved in the region of cooling.

We believe that by including a factor ("thickness of cortex"), our equation will be of general character, i.e. it will apply to other aminal species as well. For the equation, to be correct, it is necessary to know the thickness of the cortex. If we want to determine the temperature within this depth, the equation referring to the cortex should be applied. In the case of determining the temperature of a deeper layer the equation referring to the white matter should be used. This latter includes the first equation as well.

As there is no essential difference in the local blood flow in the brain of different mammals, it can be assumed that by knowing the temperature of the brain surface and by taking into account the thickness of the cortex our equations can be applied to human beings as well.

Appendix

A physical model and a mathematical relation have been established, by which, knowing the temperature of the brain surface, the temperature at different depths of the brain can be calculated.

The general form of the differential equation expressing the heat distribution in the brain. Let us consider the thermal energetical equilibrium of a site of the brain surrounded by a surface S, and of volume V. Let the specific heat of this site be C, and its density ρ. To change the temperature of site V in dt time with $d\vartheta$

$$dQ = dt \int_V c\rho \frac{\gamma \vartheta}{\gamma t} dV \tag{1}$$

a quantity of heat (Equ. 1) is necessary. The volume change and the specific heat change due to the temperature change of the investigated brain will be neglected in the following.

The dQ quantity of heat may originate from two sources. It may be produced by heat source q_v in site V, or may be carried to the site by heat conduction through S surface surrounding the site. Therefore it may be written:

$$dQ = dQ_q = dQ_S \tag{2}$$

where dQ_q is the quantity of heat produced by the heat source during dt time, and dQ_S is the resultant of the quantity of heat ingressing and egressing on S surface during dt time.

The components of relation (2) are, in detail:

$$dQ_q = dt \int_V q_v \, dV \tag{3}$$

$$dQ_S = -dt \int_S \bar{q} \, \overline{dS} \tag{4}$$

$\bar{q}$ in the equation is the vector of the density of the convection of heat.

With the help of relations (1—4) it may be written:

$$c \rho \, dt \int_V \frac{\partial \vartheta}{\partial t} dV = dt \left(\int_V q_V \, dV - \int_S \bar{q} \, \overline{dS} \right) \tag{5}$$

With the help of Gauss—Ostogradskii's thesis the surface integral can be transformed into volume integral:

$$dt \int_V \left(\rho \, c \frac{\partial \vartheta}{\partial t} + \operatorname{div} \bar{q} - q_V \right) dV = 0 \tag{6}$$

According to Fourier the vector of the density of the convection of heat can be written with the introduction of the factor of convection of heat, λ

$$\bar{q} = -\lambda \operatorname{grad} \vartheta \tag{7}$$

equation (6) will change in the following way:

$$\rho c \frac{\partial \vartheta}{\partial t} = \operatorname{div} (\lambda \operatorname{grad} \vartheta) + q_V \tag{8}$$

Using the usual mathematical designations after rearrangement we get:

$$\frac{\partial \vartheta}{\partial t} = \frac{\lambda}{\rho C} \nabla^2 \vartheta + \frac{q_v}{\rho C} \tag{9}$$

The partial differential equation (9) of second order gives a correct picture about the thermal conditions of each site surrounded by surface S and of volume V. Thus, it can be considered as the general differential equation of heat distribution in the brain.

By knowing the structure of the brain differential equation (9) can be solved. How correctly the heat will be distributed in reality and how it will change in time and space depend on the presumed structure and on the physical model of the brain.

The physical model of the brain. It is known that the blood supply of the grey matter of the cortex is 4 to 5 times better than that of the white matter. This means, that the grey matter produces 4 to 5 times more heat per time and volume units than the white matter serving as a basis of comparison. In other words, the intensity of the heat source of the grey matter exceeds the intensity of the heat source of the white matter. Therefore in our investigation the brain is considered as a heat source which is layered and uniformly distributed in layers.

In the following the thickness of the grey matter will be marked with H; the quotient of the intensity of the heat source of the grey matter and that of the white matter with K. In the course of our derivation distance X is always measured from the surface of the brain.

The intensity of the heat source of the brain is the function not only of the distance measured from the surface but also of the actual temperature. The intensity of the heat source of both white and grey matters increases in a monotonous way with decreasing temperature, and becomes zero at those sites where the temperature has reached the normal temperature level of the brain.

This is explained in the following way: According to the above said, in an intact skull the brain has a determined level of thermal energy. After craniectomy and after placing the thermode on the brain surface, the earlier energy

equilibrium becomes upset. Thermal energy is passed over to the thermode by the surface matter of the brain and during this it cools down. The lower is the temperature to which the brain cools down, the more thermal energy is liberated and flows towards the thermode. This convection of thermal energy continues not only during the cooling down but also during the new thermal equilibrium developed, during the steady-state condition as well. Even in this case the cooled brain is supplied by blood of the same temperature as before from the environment. Thus, the continuity of the convection of heat is ensured. Most of the thermal energy is supplied by those parts of the brain which are nearest to the thermode and are most cooled down, less is supplied by the deeper situated brain tissues which are warmer. In this way the sites of normal temperature do not produce heat any longer, and thus, the temperature of the site does not increase above the normal temperature level, either.

In the following we suppose that the intensity of the heat source is a linear function of the temperature

$$q_v(X;\vartheta) = \begin{cases} Kq_0(\vartheta m - \vartheta) & \text{if} \quad 0 < X \leq H \\ q_0(\vartheta m - \vartheta) & \text{if} \quad X > H \end{cases} \tag{10}$$

where q_0 is the intensity of the heat source in the white matter
ϑ_m is the normal temperature of the brain
ϑ is the actual temperature

With the help of the thermode let us stipulate for a temperature ϑ_f on the surface of the brain (this means a boundary condition of the first kind for differential equation (9)) and find the temperature values at different depths of the brain in steady-state condition of the site to be investigated.

If the applied thermode is large or if the investigations are limited to small depths in comparison with the dimension of the thermode, the changes in temperature can be considered as demodulated and the partial differential equation (9) can be described as

$$\frac{d\vartheta}{dt} = \frac{\lambda}{\rho c}\frac{d^2\vartheta}{dX^2} + \frac{1}{\rho c} q_v(X;\vartheta) \tag{11}$$

Steady-state temperature distribution in the brain. Differential equation (11) can be solved by knowing the function (10) describing the distribution of the heat source in the case of given initial conditions.

The first initial condition should express that the temperature of the brain surface corresponds to the temperature of the thermode. The second initial condition should state: at high depths from the brain surface the temperature reaches its normal level. The exactness of the initial conditions have been proved

by measurements in experiments performed on animals. The initial conditions can be expressed in a mathematical way as follows:

$$\vartheta_{(0)} = \vartheta_f \quad \text{and} \quad \vartheta(\infty) = \vartheta_m \tag{12}$$

Let us suppose for the solvation of differential equation (11) that the grey matter and the white matter show equal thermal conduction characteristics. Thus, at the interface between the grey and the white matter at a depth H the equation is

$$\frac{d\vartheta_1}{dX}\bigg| H = \frac{d\vartheta_2}{dX}\bigg| H \tag{13}$$

where ϑ_1 is the temperature of the grey matter,
ϑ_2 is the temperature of the white matter.
Under given conditions the solvation of differential equation (11) for the brain of a structure as established in equation (10) in the case of a steady-state distribution of heat is the following:

$$T = T_f e^{-m_1 X} + (T_H - T_f e^{-m_1 H}) \frac{\mathrm{sh} m_1 X}{\mathrm{sh} m_1 H} \quad \text{if} \quad 0 < X \leq H \tag{14}$$

(in the grey matter) and

$$T = T_H e^{-m_2 (X-H)} \quad \text{if} \quad X > H \tag{15}$$

(in the white matter), where

$$T_H = \frac{m_1 T_f e^{-m_1 H} (1 + \mathrm{th} m_1 H)}{m_1 + m_2 \mathrm{th} m_1 H} \tag{16}$$

$$m_1 = \sqrt{\frac{K q_0}{\lambda}} \quad \text{and} \quad m_2 = \sqrt{\frac{q_0}{\lambda}} \tag{17}$$

Let us summarize the designations occurring in these equations

T	$= \vartheta_m - \vartheta$	The magnitude of the temperature decrease in °C
T_f	$= \vartheta_m - \vartheta_f$	The temperature decrease in the brain surface in °C
T_H	$= \vartheta_m - \vartheta_H$	Temperature decrease at the interface in °C
ϑ_H		Temperature developing at the interface in °C
H		Thickness of the grey matter in mm
X		Distance measured from the brain surface in mm
K		Intensity factor, the quotient of the intensity of the heat source in the grey matter and that in the white matter
q_0		Intensity of the heat source in the white matter
λ		Thermal conduction factor of the brain
m_1, m_2		Auxiliary amounts

The prove of the exactness of the calculation conveyed in the previous section the results of measurements performed in a series of animal experiments have been evaluated.

During the experiments 31 coolings were performed on the cortex of dogs by a thermode 18 mm in diameter. The distribution of heat was measured by a thermistor at depths of 0, 1, 2, and 5 mm, respectively. Taking values of $K =$

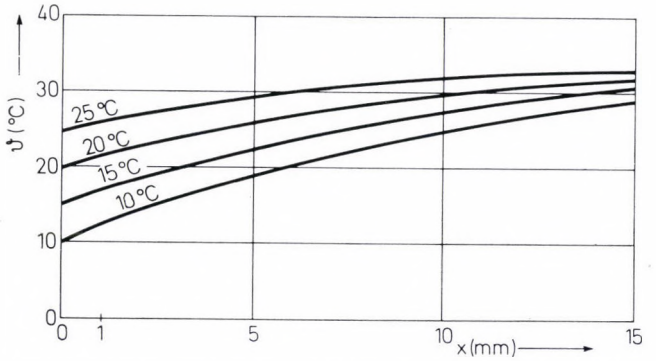

Fig. 4. Steady-state temperature distribution calculated with $K = 5$; $m_1 = 0.15$/mm; $m_2 = 0.067$/mm; $H = 2$ mm values as a function of the distance measured from the brain surface

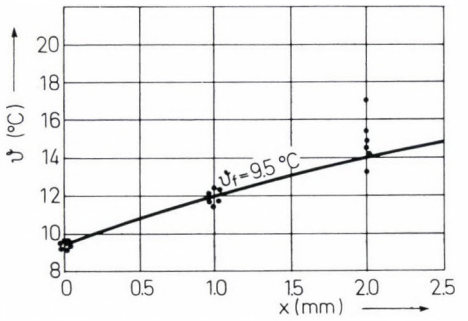

Fig. 5. The calculated temperature distribution describes exactly the temperature conditions developed in reality at small depths in relation to the 18 mm diameter of the thermode

$= 5$ and $H = 2$ mm characteristic for dogs into consideration $m_1 = 0.15$/mm and $m_2 = 0.067$/mm were obtained. Fig. 4 shows the steady-state distribution of heat calculated from these data with the help of Equations (14—16).

In Fig. 5 the temperature values measured in the experiments and those corrected with a systematic error are compared to the heat distribution calculated mathematically from the proposed relation.

It can be seen in Fig. 5 that the calculated and measured results show a good agreement down to depths (about 1.5 to 2 mm) which are small enough in relation to the 18 mm diameter of the thermode.

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Thickening of the Striated Muscle During Contraction

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An analysis was performed on the thickening of the striated muscle of the frog during contraction. By the help of a mechanoelectrical transducer system the shortening and the thickening were simultaneously recorded. It is concluded that the thickening of the muscle shows a time course similar to that of the shortening, that is, the thickening can be approximately described by a sine function.

Numerous studies in the literature have been concerned with the mechanical activity of the striated muscle, mainly as regards the changes in length or in force development during contraction, but the question about the change of thickness during contraction seems to have been neglected. In connection with the thickening only a few short references can be cited, mainly from classical works (Frey, 1909; Dittler, Oinuma, 1911), in which, however, no attention was paid to the detailed analysis of the thickening.

The problem seemed to be an interesting one, because

a) the absolute force exerted by the muscle is proportional to the thickness (e.g. Ernst, 1963), and this latter increases during shortening;

b) the thickness of the muscle increases at active hypertrophy as well;

c) the investigation upon thickening can yield informations about the time relation between excitation and mechanical activity;

d) from morphological point of view, the analysis of the thickening may shed some light upon the problem, how the contraction can be interpreted in terms of the microscopic and submicroscopic structure of the muscle;

e) from practical point of view, the difference existing in the thickness of the muscle in its resting and shortened state may play an important role in the great achievements in sport.

Methods

The muscles used throughout this work were gastrocnemius, semimembranosus and sartorius of the frog *(Rana esculenta)*. The muscles were prepared immediately before the experiments at room temperature, and were kept immersed

for a short time in Ringer's solution. During the experiments only the first few twitches or tetani were considered as physiological.

To record the thickening of the muscles studied, a special transducer system was developed. Recording of thickening alone gives an incomplete picture about the mechanical state of the muscle, so we decided to record simultaneously both

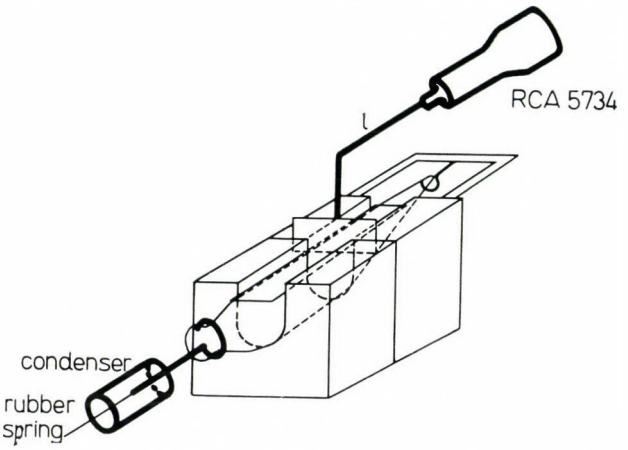

Fig. 1. Schematic diagram of the apparatus used for making simultaneous records of shortening and thickening

the shortening and thickening of the muscle to compare their time course during contraction under the same conditions. The shortening was recorded by connecting the muscle to a capacity transducer. Details about this type of transducer were already published in a previous work by Mórocz-Juhász and Örkényi (1967). Thickening of the muscle was recorded by coupling a light lever (*l*) to an RCA 5734 transducer tube.

The experimental arrangement can be seen in Fig. 1. The muscle was placed in a special holder made from plexi-glass. In the holder a little groove in the form of a semicircle was milled. This was so tight that the muscle laying in it could thicken only in one direction. To the lower end of the lever (*l*) two light, but rigid brass plates were mounted, which were soldered perpendicular to each other, as can be seen in Fig. 2. The horizontal one (*h*) lad on the surface of the muscle and covered about one third of it, the other plate (*v*) could move in the narrow slit made in the plexi-glass muscle holder. This arrangement ensured that, on the one hand, the horizontal plate (*h*) touched the maximally thickened part of the muscle at any time during contraction, and on the other hand, the recording at the transducer tube showed the real thickening because the lever could move only vertically (both upwards and downwards) and so the movement caused by the shortening in horizontal direction was hindered.

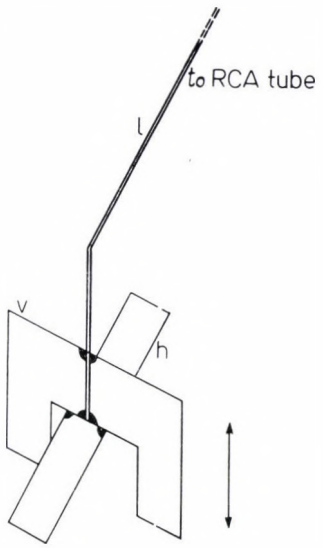

Fig. 2. Schematic diagram of the lever system

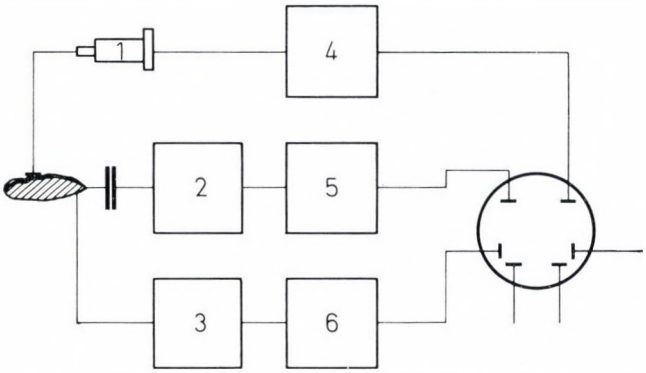

Fig. 3. Schematic diagram of the experimental arrangement. Symbols: (1) RCA transducer tube, (2) capacity transducer, (3) stimulator, (4) and (5) amplifiers, (6) sweep generator

By attaching the lever to the transducer the high resonance frequency of the tube dropped to a few hundred cycles per second. This was estimated after a light impact on the lever. Taking into account the time course of the thickening to be expected, this lower resonance frequency of the lever-transducer tube system was high enough to get a recording without any appreciable distortion.

The outputs of the capacity transducer and RCA tube were amplified and displayed simultaneously on the upper and lower traces of a double-beam oscillo-

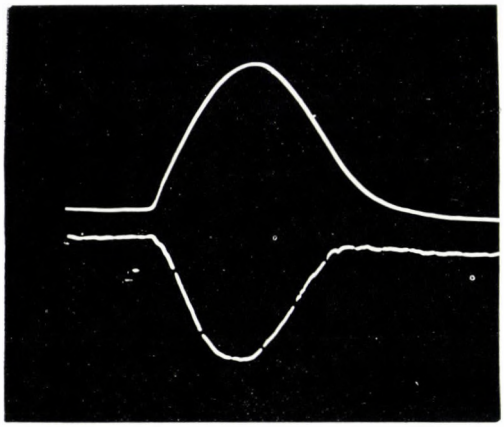

Fig. 4. Traces of simultaneous records of shortening and thickening of a frog gastrocnemius. Upper line: shortening; lower line: thickening. Time marks: 10 ms

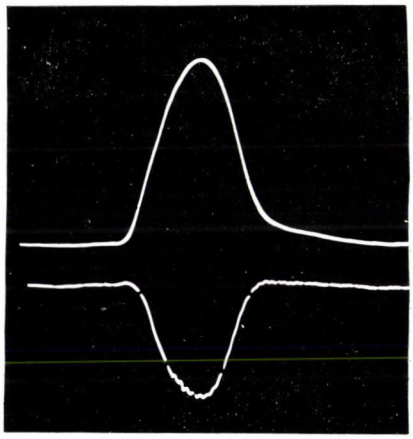

Fig. 5. Records of shortening and thickening of a frog semimembranosus. Upper line: shortening; lower line: thickening. Time marks: 10 ms

scope (see Fig. 3). The electronic circuit used to amplify the output of the RCA tube was similar in many respects to that described by Levy (1962).

All the experiments were performed on muscles in resting length. The muscles were stimulated with supermaximal rectangular pulses of 1 ms duration; in other cases, when the shortening and thickening were studied under tetanic condition, an impulse series of 0.2 or 0.4 s time duration was used with a frequency of 50 c/s. The stimulating pulses were also recorded on the CRO.

The output was photographed from the cathode ray oscilloscope. Measurements were made on the film by magnifying the original CRO deflection which permitted an exact analysis of the muscle activity.

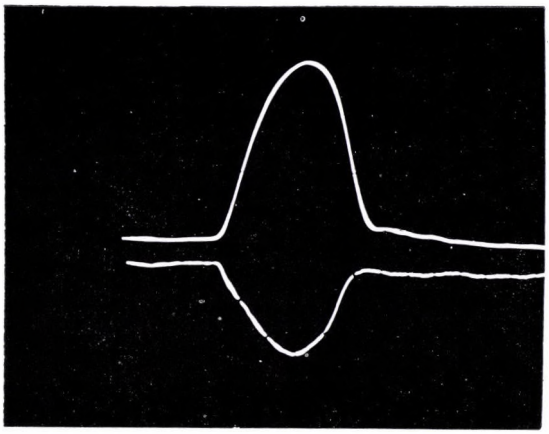

Fig. 6. Records made on frog's sartorius. Upper line: shortening; lower line: thickening. Time marks: 10 ms

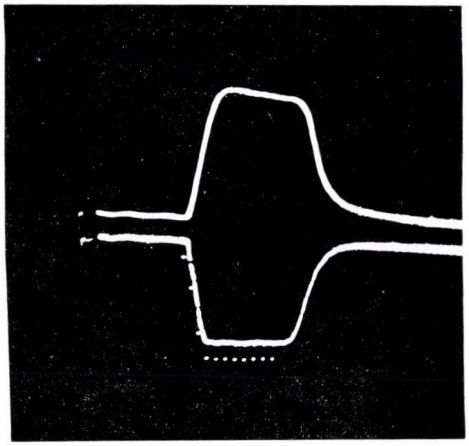

Fig. 7. Shortening and thickening of a frog gastrocnemius under tetanic condition. Upper line: shortening; lower line: thickening. The tetanus lasted for 0.2 s

Results

1) Fig. 4 shows a record of the excised frog's gastrocnemius. The upper line represents the shortening, the lower line the thickening curve. As can be seen from the trace, the thickening has a time course similar to that of the shortening, both of them reach their maximum value at the same time, and have about the same relaxing phase. The rising phase of the thickening was found to be 45 $\pm$ 4 ms on average of several determinations. Frey (1909) has reported a similar observation in the past.

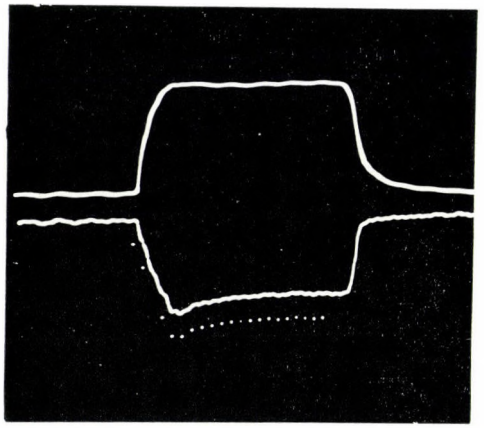

Fig. 8. Shortening and thickening of a frog sartorius during a tetanus. The tetanus lasted for 0.4 s. Upper line: shortening; lower line: thickening

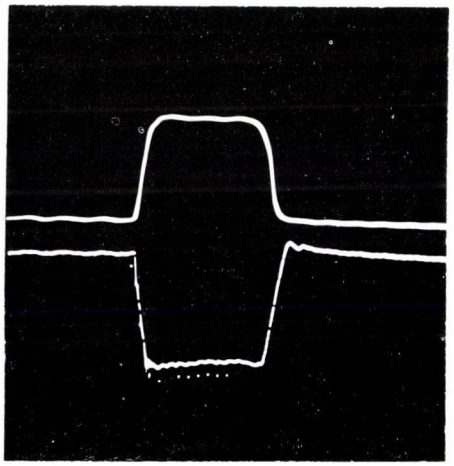

Fig. 9. Shortening and thickening of a frog semimembranosus during a tetanus. Tetanus lasted for 0.2 s. Upper line: shortening; lower line: thickening

Fig. 5 and Fig. 6 show the records of semimembranosus and sartorius of the frog. As it can be seen from the photographs, as regards the time course of the shortening and thickening curves, the same conclusions can be drawn as in the case of gastrocnemius. The experiments carried out by Brücke (1908) on sartorius showed that the time necessary to reach the maximum thickening was 43 ms, as a mean value, with fresh muscle. In our experiments we found 28 ± 5 ms for sartorius and 30 ± 3 ms for semimembranosus.

2) Fig. 7, Fig. 8, and Fig. 9 represent the simultaneous records of the shortening and thickening of gastrocnemius, semimembranosus and sartorius during tetanus. The time duration of stimulation (the single stimuli are marked on the lower line as small points) was 0.2 s or 0.4 s, respectively.

Discussion

On the basis of the magnified photographs a simple analysis was performed to get a quantitative picture about the time course of the thickening and to compare the time course of the shortening and the thickening in the case of a twitch. The result of the analysis for semimembranosus and sartorius is presented in Table 1. In the Table the values are referred to the maximum value of the

Table 1

Result of the analysis carried out on the thickening and the shortening of the sartorius and semimembranosus of the frog during a twitch

		0	$\frac{1}{5}$ T	$\frac{2}{5}$ T	$\frac{3}{5}$ T	$\frac{4}{5}$ T	T
M. sartorius	thickening	0	0.32 ±0.07	0.56 ±0.07	0.80 ±0.05	0.93 ±0.02	1
	shortening	0	0.26 ±0.05	0.58 ±0.08	0.79 ±0.05	0.93 ±0.03	1
M. semimembranosus	thickening	0	0.21 ±0.07	0.54 ±0.05	0.80 ±0.05	0.94 ±0.02	1
	shortening	0	0.25 ±0.05	0.57 ±0.06	0.80 ±0.06	0.94 ±0.02	1

shortening and of thickening; T means the duration of the rising phase. The analysis shows that there is no statistically significant difference between the rising phase of the shortening and of thickening. The test of significance was calculated by the t-test at a probability level of $P = 0.05$.

Therefore, it can be calculated that the time course of the thickening can be described in good approximation with the same $f(t)$ function, as the shortening. On the basis of earlier data and measurements it has already been reported (Ernst, 1963; Belágyi, Örkényi, 1967) that the shortening of the frog's muscle fulfils nearly the sine function for a twitch. It follows therefore, that

$$\frac{q}{Q} = \sin \frac{\pi}{2} \frac{t}{T}$$

where q is the momentary thickening, Q the maximum thickening, T the duration of the rising phase, t the time corresponding to q.

The sine function and the relative value of the thickening are compared in Table 2 for the muscles studied. It appears that the data of the experiments are smaller than the theoretically calculated values. This difference can be explained probably by the fact that our supposition is not exactly correct, that is, the muscle could thicken in the groove not only in the direction of the lever of the RCA tube, because of its non-identical cross section, but in other direction as well, and so the corresponding deflections are smaller than the expected ones. This is particularly true for m. gastrocnemius.

The result obtained for the time curve of the thickening can be interpreted by a simple model in the following way: Suppose that upon shortening the muscle behaves similarly to an elastic material, and therefore, it can be expected that

Table 2

Comparison between the sine function and the relative values of the thickening with the different muscles

Time	0	$\frac{T}{5}$	$2\frac{T}{5}$	$3\frac{T}{5}$	$4\frac{T}{5}$	T
Sine function	0	0.31	0.59	0.81	0.95	1.00
M. gastrocnemius	0	0.24 $\pm$0.04	0.49 $\pm$0.04	0.76 $\pm$0.04	0.94 $\pm$0.01	1.00
M. sartorius	0	0.32 $\pm$0.07	0.56 $\pm$0.07	0.80 $\pm$0.05	0.93 $\pm$0.02	1.00
M. semimembranosus	0	0.21 $\pm$0.07	0.54 $\pm$0.05	0.80 $\pm$0.05	0.94 $\pm$0.02	1.00

its volume remains constant during contraction. Now, assuming that the muscle in the holder would change its thickness in the groove only upwards it can be easily obtained by simple calculation, that the transducer tube's deflection, which is proportional to the thickening is proportional to the shortening as well, that is

$$\Delta s \sim \frac{\Delta r}{L_0}$$

where Δs is the deflection at the end of the lever, Δr is the shortening corresponding to Δs at a given time of t, L_0 is the resting length of the muscle.

As regards the thickening during a tetanus, as can be seen from the photographs, the time course of the thickening of all three muscles is quite similar to that of shortening. Ernst (1963) has proposed in his monograph that a tetanus

can be considered as superposition of single twitches. According to the experimental results obtained with tetanus, we think, that this superposition can be expected for thickening during a tetanus as well.

Several models have already been proposed to interpret the mechanical activity of the muscle during contraction by structural changes. In one of these, analysed in detail by Huxley and Hanson (1954), the shortening is generated by the sliding of the thin and thick filaments past each other assuming that the over all lengths of both filaments remain constant.

Our result, however, shows a thickening simultaneous with the shortening. This is probably valid not only for the whole muscle, but for a single fiber as for the basic contractile unit, as well (Kominz, 1968). The thickening of the structural elements during contraction, especially of the A-band, was observed by conventional light microscope measurements on glycerol-extracted muscle fibers treated with ATP in the work of Huxley and Hanson, cited above. Therefore, the thickening which is simultaneous with the shortening during contraction can be interpreted by assuming conformational changes taking place in the macromolecules of the filaments and an interaction between the molecules of the filaments and surrounding medium and not only the sliding of the filaments.

Acknowledgement

Authors are indebted to Professor E. Ernst for calling attention to this topic and for his help during the work.

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Frequent Action Potentials of Frog's Nerve and Muscle

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Experiments were performed on frog's ischiadicus-gastrocnemius preparation to examine the hypothesis according to wich the motor nerves are also able to transform the constant course of d.c. into frequent impulses of excitation (Frey, 1883; Ernst, 1966, 1967). Our experiments demonstrate that the response of the preparation to an indirect stimulus of d.c. consists of a muscle tetanus and of the trains of action potentials developed by both the nerve and the muscle, and further, that the trains of action potentials recorded on the nerve and the muscle become simultaneously longer with increasing duration of the rectangular pulses. In our opinion the frequent action potentials developed by the nerve and the muscle are related to coding the stimulus into excitation.

Introduction

Present investigations have strated out of the hypothesis (Frey, 1883) according to which motor nerves are able to transform a d.c. stimulus into frequent excitatory impulses. Several papers have been published on the problem of frequent excitation evoked by different stimuli. In the case of the d.c. stimulation of the frog's ischiadicus, Fessard (1936) has demonstrated a train of action potentials having rarely more than four waves resembling a rapidly damped oscillation. Erlanger and Blair (1936) have also studied the repetitive response of the frog's nerve connected with the effects of electrotonus. Katz (1936) has reported on a repetitive response which had been produced by constant current in the nerves excised from cooled frogs or in nerves soaked in Ca-free solution. Similar results concerning the repetitive action of the different nerves were obtained by Fessard (1936) on crayfish non-myelinated nerve, by Skoglund (1942) on cat's motor and sensory roots and by Hodgkin (1948) on sensory axon of Carcinus maenas.

The frequency modulation brought about by excitable tissues (e.g. Ernst, 1966) and the role of simple nerve fibres generating and transmitting different frequencies can be significant with respect to communication of information within the organism (Ernst, 1967; in press). It seemed worthwhile, therefore, to test Frey's hypothesis by experiments which constitute a continuation of our former biocybernetical studies on stimulus-excitation processes (Biró, Királyfalvi, 1965, 1966).

Methods

The experiments were performed with isolated ischiadicus-gastrocnemius preparations from frogs *(Rana esculenta)* of autumn, winter and spring. The preparations were placed in a moist chamber. The experiments were carried out at room temperature.

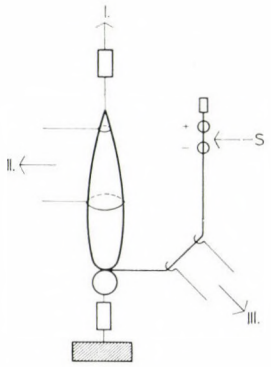


Fig. 1. Sketch of the arrangement of experiment

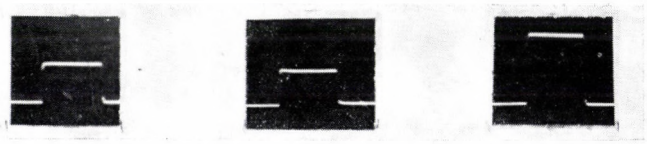
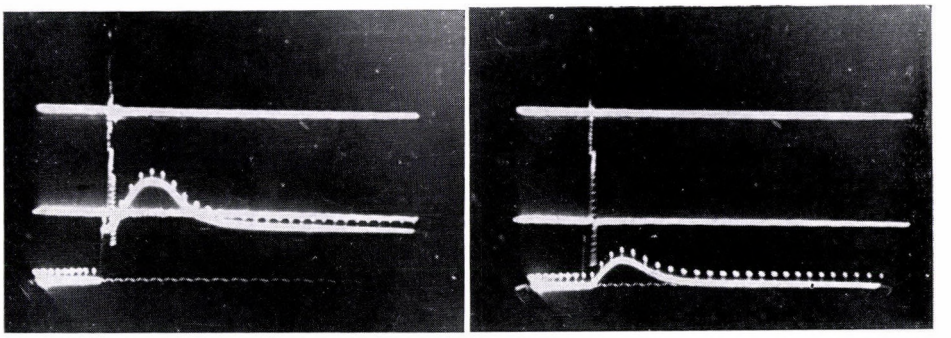


Fig. 2. Calibration impulses: 10 mV (a) and 1 mV (b) for channel III and 100 mV (c) for channel II

Three pairs of electrodes were connected with the preparations: two non-polarizable Ag-AgCl-Ringer electrodes for stimulation, two platinum electrodes 1 mm in diameter and two platinum electrodes 0.1 mm in diameter for recording the action potentials of the nerve and the muscle, respectively. Fig. 1 shows the position of the electrodes with respect to each other and to the preparation. The proximal end of the muscle stretched to its resting length was fixed, the other end of it was connected with a mechanoelectric transducer (Mórocz-Juhász, Örkényi, 1967) recording the muscle contraction (Fig. 1).

A triple-beam oscilloscope was used in our experiments. The following three signals were recorded by the lower beam (channel I): muscle contraction, time markers and stimulus. The action potentials of the muscle and the nerve were recorded by the middle beam (channel II) and the upper beam (channel III), respectively. The calibration impulses for the channels are shown in Fig. 2.



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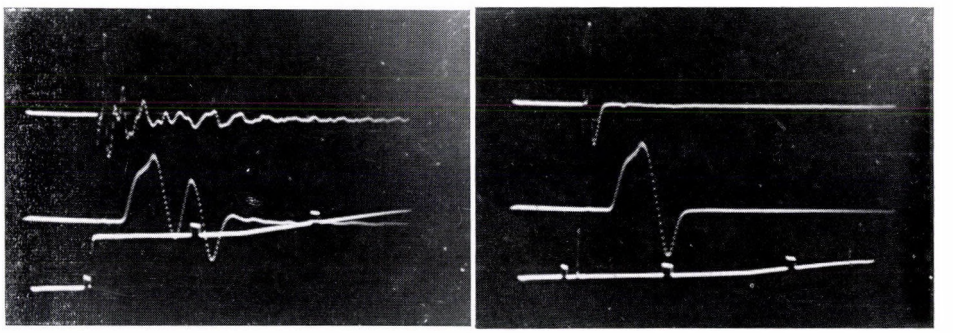

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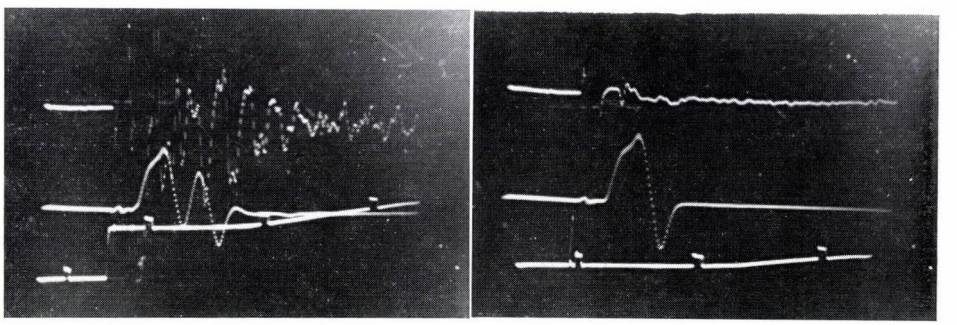
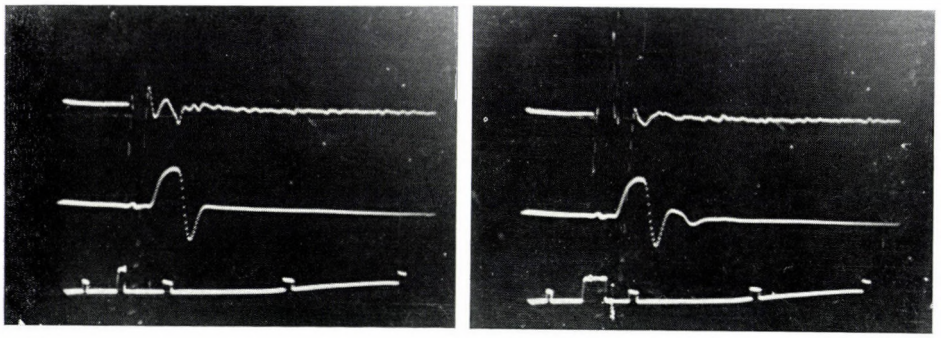


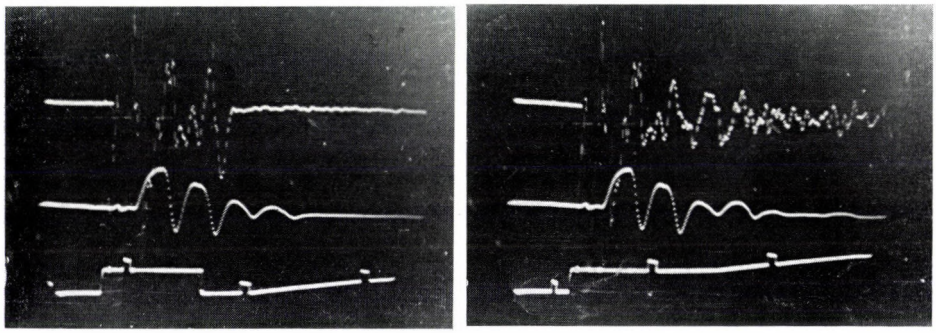
3/Ac 3/Bc

Fig. 3. Oscillograms of the action potentials of the nerve (upper beam), the muscle (middle beam) and the muscle contraction (lower beam) in the case of d.c. stimulus (A) and a rectangular pulse of 0.1 ms (B). Amplification of the nerve action potentials in pictures *c* is ten times larger than in pictures *a* and *b*. Stimulus amplitude: 1.2 V. Time markers: 10 ms

The responses of the preparation were elicited by stimulation of the nerve with supramaximal stimuli delivered by a stimulator which produced both the d.c. stimuli and the rectangular pulses of different amplitudes (0.01—100 V)



4/a 4/b



4/c 4/d

Fig. 4. Oscillograms of the action potentials of the nerve (upper beam) and the muscle (middle beam) and the stimulus together with the beginning of contraction (lower beam) in the case of rectangular stimuli of 1 ms (a), 3 ms (b), 10 ms (c) and 30 ms (d). Stimulus amplitude: 1 V. Time markers: 10 ms

and durations (0.04—30 ms). In the case of d.c. stimulation the closure of descending d.c. was used because under such conditions the excitation originated at the cathode and the excitation travelling towards the muscle was not influenced by the developed anelectrotonus.

Results

In part of our experiments a comparison was made between the effects of an indirect supramaximal[1] rectangular pulse of 0.1 ms and a d.c. stimulus of the same voltage. In the case of a d.c. stimulus the oscillograms showed that the trains of action potentials were developed by both the nerve and the muscle and that the trains were accompained by a muscle tetanus (Fig. 3).

[1] If the amplitude of rectangular pulse is increased further, the amplitude of the action potentials led from the nerve and the muscle and the size of muscle contraction are not increased.

In another part of the experiments the trains of action potentials recorded on the nerve and the muscle became simultaneously longer with increasing duration of the rectangular stimuli (Fig. 4).

Results shown in Fig. 3 and 4 support Frey's hypothesis about the ability of the nerve to transform a constant stimulus into frequent impulses of excitation.

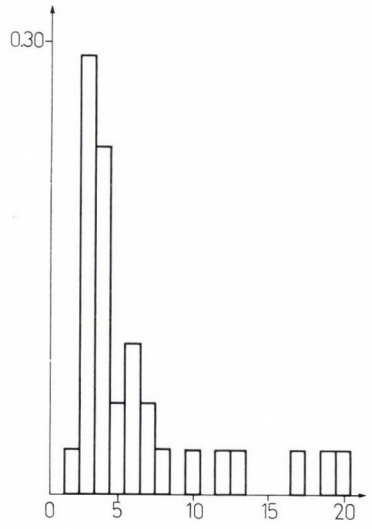


Fig. 5. Histogram of the numbers of the nerve action potentials

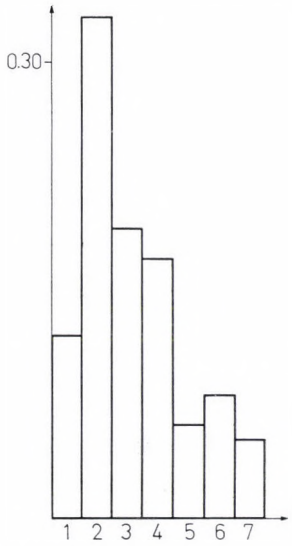


Fig. 6. Histogram of the numbers of the muscle action potentials

Table 1

Distribution of the numbers of the nerve action potentials in the different experiments

Number of the nerve action potentials	1	2	3	4	5	6	7	8	9	10
Number of experiments	—	1	9	7	2	3	2	1	—	1
Relative frequency		0.03	0.29	0.23	0.06	0.10	0.06	0.03		0.03
Number of the nerve action potentials	11	12	13	14	15	16	17	18	19	20
Number of experiments	—	1	1	—	—	—	1	—		1
Relative frequency		0.03	0.03				0.03		0.03	0.03

The number of action potentials of the nerve and the muscle in the different experiments was found to be different. (In the case of nerves only those action potentials were taken into consideration whose peak to peak amplitude was greater than 0.5 mV.) The number of action potentials and their relative frequencies are shown in Table 1 and Fig. 5 for the nerves, and in Table 2 and Fig. 6 for the muscles.

Table 2

Distribution of the numbers of the muscle action potentials in the different experiments

Number of the muscle action potentials	1	2	3	4	5	6	7
Number of experiments	8	21	12	11	4	5	3
Relative frequency	0.12	0.33	0.19	0.17	0.06	0.08	0.05

The frequencies of the action potentials of nerve and muscle have been calculated by dividing the number of action potentials by the duration of their train. The quantitative data of the experiments as well as the correlation coefficients concerning the corresponding data of the nerves and the muscles are given in Table 3.

Table 3

Values of the means and the standard deviation of means of the experimental results and the correlation coefficients

	Nerve	Muscle	Correlation coefficient
Number of action potentials	6 ± 1	3 ± 1	0.75 ± 0.08
Duration of the trains (ms)	10 ± 1	21 ± 2	0.65 ± 0.12
Frequency of action potentials (Hz)	613 ± 21	186 ± 7	0.51 ± 0.15

Discussion

According to Fig. 3 and 4 the excitation of the nerve and the muscle to d.c. or rectangular pulses of suitable duration took a periodic course. The question may arise in connection with the interpretation of these oscillograms that the train of nerve action potentials was formed by the successive registration of the individual action potentials of the nerve fibres having different conduction velocities (fibres A, B, C, etc.) in the nerve trunk. However, this interpretation

contradicts the results that the trains of action potentials were generated only by d.c. and rectangular pulses of suitable duration, but not by a supramaximal rectangular pulse of 0.1 ms which had to stimulate all the fibres of the nerve trunk.

It is remarkable that the amplitudes of the succeeding action potentials decreased. Provided the nerve fibres have different accommodation, the recorded action potentials, which have to be the result of the summarized action potentials belonging to the different nerve fibres in the trunk, may be formed by the trains of action potentials of constant amplitude if the trains have different lengths. In addition, it must be taken into consideration that the excitatory processes of the nerve fibres are not perfectly synchronized (Fessard, 1936). To clear up these problems experiments performed on single nerve fibres are required.

The question of analogy between the excitation and the damped oscillation has recently been studied by Michalov (1967) in connection with the decrease of the amplitudes of action potentials. It is certain that such an analogy can be raised under some circumstances. However, according to the oscillograms shown in Fig. 4 it appears that the nerve can respond with action potentials until the end of the stimulus, and if the duration of a stimulus is not too long, the further range of excitatory "oscillation" is omitted. In our opinion this phenomenon is in fact not a damped oscillatory motion but it seems to be an accommodation process of the nerve.

The frequencies of the nerve and the muscle action potentials generated by a d.c. stimulus may have a bearing on the natural frequency of the different excitable tissues (Ernst, 1963). On the other hand the natural frequency of the different excitatory channels may be important from the point of view of coding or decoding in the excitatory process.

The fact that the correlation coefficient concerning the frequencies of the nerve and the muscle action potentials[1] is not more than $c = 0.51 \pm 0.15$, may be caused by the different experimental conditions (e.g. seasonal distribution of the frogs or different room temperatures). These may have a different influence on the rhythmic features of the nerve and the muscle and possibly on the motor end-plate as well. However, the value of this correlation cofficient can also serve as a clue for the independent rhythmic behaviour of the muscles according to which the muscle cannot be regarded just as a passive realizer of the orders given by the central nervous system (Klinghardt, 1951) and according to which the "muscle introduces major modifications into the signals which it handles" (Partridge, 1966). In connection with this problem it would be interesting to examine what kind of relations exist among the frequencies of the action potentials of the nerve and the muscle and the motor end-plate in the case of stimuli of different frequencies and in the case of stimuli of frequencies changing continuously. The

[1] It should be remembered that the action potentials led from the nerve trunk contain the responses of afferent fibres, too, which are irrelevant to the action potentials of the muscle.

determination of the connection between these frequencies would be important with respect to the investigation of information transmission from the nerve to the muscle or of the coding performed by the motor end-plate (Ernst, 1961).

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The author is indebted to Professor E. Ernst for raising the problem and for his encouragement during the work.

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The Mechanism of Coding Information in Nerves*

Excitation as an Electron Process III

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1. Frequency modulation in nerves. — 2. Frequency modulation as an electron process — performed by the substance of the nerve. — 3. Phenomena of electrotonus as an electron process — 4. Mechanism of frequency modulation. — 5. Outlook and Summary.

1. Frequency modulation in nerves

This topic has been treated in earlier papers (Ernst, 1966; Ernst et al., 1967; Biró, 1968), thus it will now be delt with but shortly. These papers quoted many authors and described data obtained also in our own laboratory. All these experimental results can be summarized as follows: Frequent spikes appear in sensory nerves as a continuation of the receptor or generator potential having been transformed from d.c. into a train of electric impulses. Similar phenomena could be found in motor nerves; furthermore, we succeeded in demonstrating that, after being sensibilized, motor nerves were able to produce frequent action currents even when stimulated with square pulses of the duration of 0.1 ms.** The phenomenon, i.e. the transformation of a d.c. impulse into a train of spikes in sensorimotor nerves, was considered as an analogy to the frequency modulation known in physics.

The fact that frequency modulation takes place in the nerve means, in the sense of the information theory, that a nerve is not only a "passive" channel in which information is propagated, but it also plays a very important "active" role. Viz., the nerve having received information mediated by some signal (receptor potential, electric stimulus, etc.) immediately transforms it into information

* Part of a lecture delivered in the Biological Section of the Hungarian Academy of Sciences at the 50th anniversary of the Great October Socialist Revolution.

** Besides the publications on frequent spikes in motor nerves quoted in my earlier papers, the following should still be mentioned: Jasper et Monnier (1933), Arvanitaki (1939), Monnier et Coppée (1939), Erlanger and Blair (1935).

consisting of impulses of different frequencies. Only these frequent impulses are understandable for the centre, therefore this process of frequency modulation in nerves means that *nerves in general are able to code the information* before propagating it.

2. Frequency modulation (coding) as an electron process

As to this conception our earlier paper (Ernst, 1966) mentioned the fact that procaine, while blocking propagation and abolishing the train of frequent impulses in sensory nerves, does not hinder the production of the receptor potential. When exposing this effect we referred to the electronic structure of procaine,

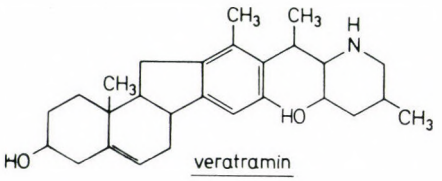

Fig. 1. Chemical structure of veratrine

the blocking effect of which can be multiplied by substituting the atom of S for the carbonyl-O. Both effects have been explained by the very mobile electrons of these compounds*. This blocking effect of novocaine has been demonstrated also on motor nerves (Gross, 1910; Herr, 1952).

According to this conception the blocking effect of procaine is ascribed to an electron process between this compound and the nerve, preventing the production of frequency modulation. This hypothesis finds substantial support in the fact that also veratrine is able to exert a blocking effect on nerves (Fleckenstein, 1951). Fig. 1 showing the chemical structure of veratrine simultaneously demonstrates that this compound contains a number of π electrons, the great mobility of which corroborates our conception as described above.

Besides, experimentally, veratrine produced a still more important phenomenon. Namely, both these compounds (novocaine, veratrine) while acting separately on nerves exert their blocking effect, but when they act simultaneously, fail in their blocking effect, and the nerves conduct quite normally. On the basis

* Simultaneously I raised the question of the expectable effect of a similar compound which results if the carbonyl-O is exchanged not with S but with Se, the outermost p electron of which, after becoming a d — π electron, is still more mobile than that of the S atom. On this line the further question arises what effects are to be expected when the carbonyl-O is exchanged with Te or Po.

of this fact our hypothesis sounds: The electron process of frequency modulation is produced by the substance of the nerve, on the one hand, and the easily mobile electrons of the compounds (mentioned above) reacting upon some nerve substance (aneurine? Muralt, 1958) prevent the process of producing spikes, on the other. But when both these compounds are simultaneously present, a reaction consisting of a donor-acceptor process* takes its course between those two compounds, thus they are unable to act also upon the nerve substance and to exert the blocking effect.

This conception emphasizing the (electron) donor-acceptor process taking place in the nerve substance (axoplasma) neglects the hypothesis of the migration of ions through the axolemma. This latter hypothesis should explain frequent spikes of 300—500 per second, or even greater frequencies recorded on nerve and muscle. Furthermore, even larger frequencies over 2000 per second have recently been described (Hagiwara, et al., 1965) for the electric organs of some fishes. In view of these facts the membrane-ion hypothesis should explain the time relations of the reversible migration of ions within a fraction of a ms *inside organic substance*. For comparison a few data on mobilities should follow: The mobility of ions (in order of magnitude) is 1 Å per 0.1 ms per 1 Vcm^{-1} in a *very dilute solution;* the mobility of electrons *in solids* under the conditions discussed here is 10^4—10^5 times greater.*

3. Phenomena of electrotonus as an electron process

Antecedently, the series of spikes in nerves have been considered as the result of the electron process of frequency modulation performed by the axon; further, the blocking effects of novocaine and veratrine have been described also as an electron process between electrons of these compounds and those of the axon. According to this conception the substance of the axon — due to its *mesomorphous*, paracrystalline structure — is able to produce electron processes similarly to solids of crystalline structure (Muralt, 1946).

a) Curve of voltage-current. Accepting this supposition one naturally arrives at the question about the basic nature of other electric functions of nerves, among them the phenomena of the well-known electrotonus. On this line one is led to the assumption that some characteristics of the electrotonus should be considered as analogies to those of crystalline solids. Fig. 2 shows the curve of the voltage-current for nerve (Hodgkin, 1938), Fig. 3 that for muscle (Katz, 1947); both curves demonstrate that linearity between the two values discontinues beginning from a certain point. By this, one is reminded of similar data obtained on certain crystalline solids showing the well-known negative resistance in which, of course, electrons are implicated (see also Wei, 1967).

* The electron structures of all these compounds should be clarified.

** Other fields of biology are also suitable for discussing the probability and usefulness of the electron- versus the membrane-ion theory of excitation.

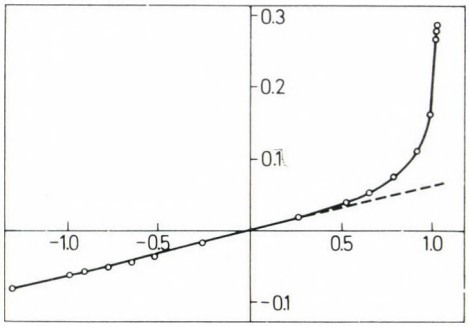

Fig. 2. Curve of voltage-current in nerve

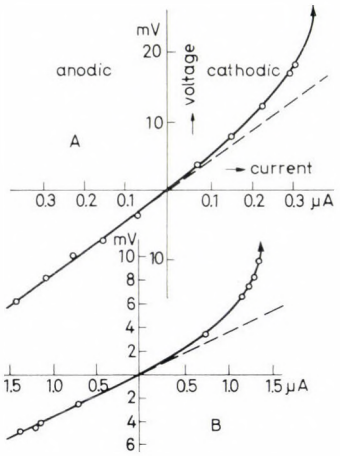

Fig. 3. Curve of voltage-current in muscle

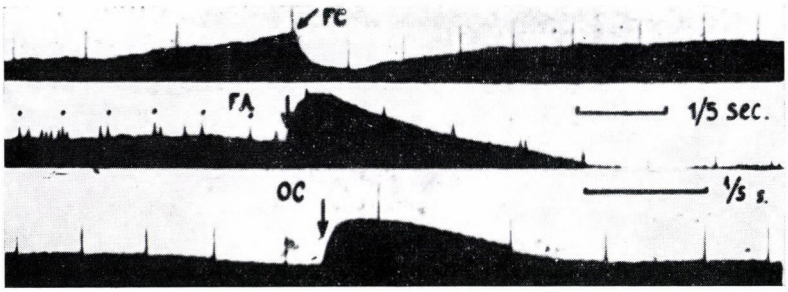

Fig. 4. Series of spikes; frequency increases in catelectrotonus, decreases in anelectrotonus

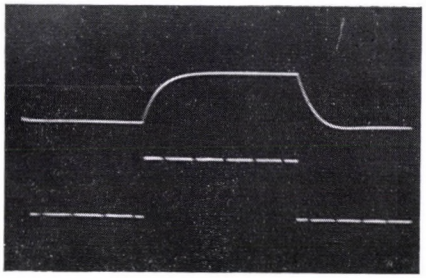

Fig. 5. Curve of output-time in an electric oscillatory equipment

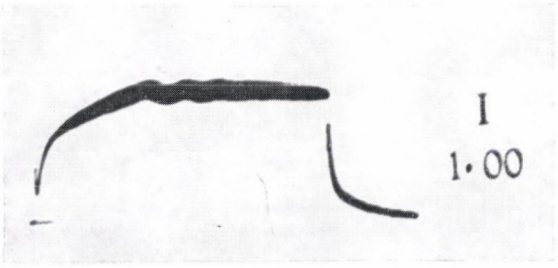

Fig. 6. Voltage-time curve in nerve

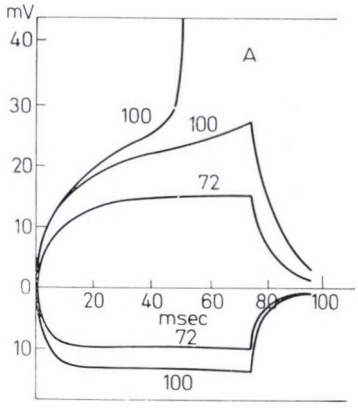

Fig. 7. Voltage-time curve in muscle

b) Rectification is known as a very important characteristic of crystalline solids; in view of this, it seems to be of a special interest that also nerves in the state of electrotonus have the ability to rectify current (e.g. Cole, Curtis, 1941). The importance of this discovery becomes more evident in the light of further experimental results demonstrating that also the rectifying effect — similarly to

frequency modulation — can be blocked by cocaine or veratrine (Guttman, Cole, 1941). Thus, the assumption of the electronic nature also of the electrotonus seems to enlarge our hypothesis discussed in this paper.

c) What has been discussed above shows that *frequency modulation and electrotonus are closely related functions* based identically on electron processes. Fig. 4 demonstrates, indeed (Fessard, 1936), that — in a series of spikes brought about on a nerve treated with NaSCN — the frequency increases in catelectrotonus and decreases in anelectrotonus.

d) In a paper (Királyfalvi, in press) an electronic control system which is able to produce electric oscillations is described. Fig. 5 shows the curves of input-output of this equipment: Figs 6 and 7 present the curves of the *voltage-time* relation in electrotonus taking place in nerve (Hodgkin, 1948) and in muscle (Katz, 1936), respectively. The similarity of these curves seems to be noteworthy.

4. Mechanism of frequency modulation

Since the series of spikes due to frequency modulation can be considered as electric oscillations the mechanism of frequency modulation in nerves should be explained on the analogy of physical equipments performing such a function,

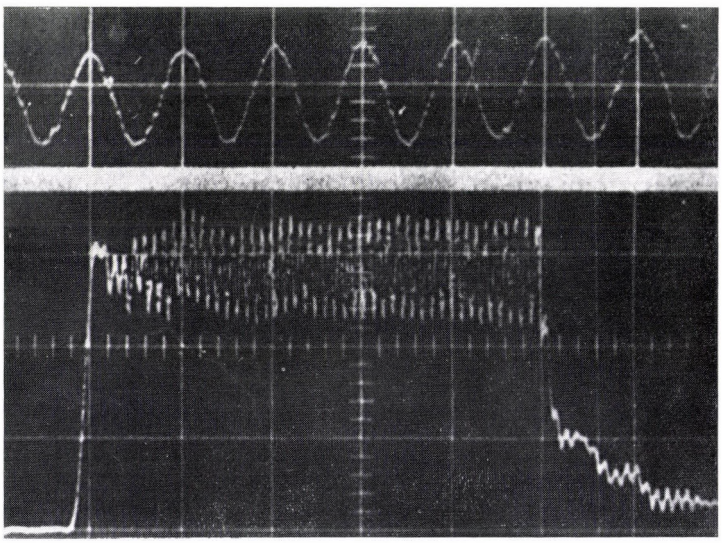

Fig. 8. Gunn-effect

Neglecting all such physical equipments containing different units (capacity, resistance, inductance, etc.) we remember some recent papers dealing with electric oscillations. Among them Gunn's discovery seems to be the most important

(Gunn, 1963; 1964): A wafer prepared from GaAs only 200 μ thick was put in an electric field of some thousand Vcm^{-1} in consequence of which frequent electric oscillations of some 10^9 Hz could be taken off from it (Fig. 8)*. And that takes place due exclusively *to the crystalline structure and the microscopic thickness of the wafer.*

Namely, electrical oscillations could till recently be produced only if the equipment contained some parts of different electric properties (e.g. capacity, etc.). Therefore in the case of nerves it seemed to be rather unfounded to consider the series of spikes produced by nerves as an analogy to frequency modulation produced by physical equipments. But now, *in the light of the Gunn-effect, the fortunate possibility offers itself to search experimentally and theoretically for frequency modulation in nerves as due to electron processes.*

5. Outlook and Summary

The series of spikes as a result of frequency modulation produced by nerves seems to be an electron process; this conception is substantiated by the Gunn-effect, according to which a wafer of GaAs produces the electron process of frequency modulation. Two years after the first paper about the Gunn-effect

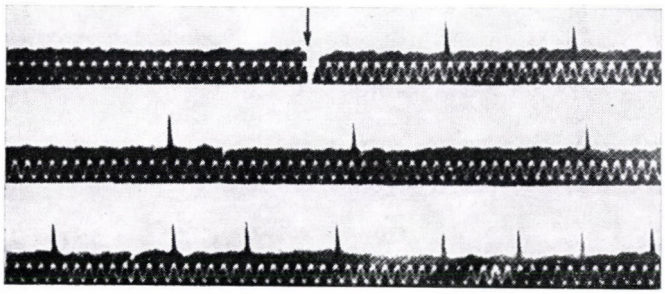

Fig. 9. Series of spikes in nerve (↓ light)

the very interesting fact was published (Nasledov, 1965) that oscillatory photodynamic effects could be found on pieces of GaP crystal (having a similar structure as GaAs).

Since both oscillatory phenomena are produced supposedly by nearly identical mechanisms, one seems to be justified in expecting photodynamic as well

* Among the antecedents of this outstanding discovery experiments may be mentioned in which pieces of Ge, InSb, InAs, GaSb of microscopic thickness produced oscillatory magneto-absorption effects (e.g. Burstein, 1956 and 1959; Zwerdling, 1956 and 1960).

as electric oscillations also in nerves.* Fig. 9 actually shows (Auger, Fessard 1933) that a nerve senzibilized with eosin produces a series of spikes due to irradiation with visible light.

Consequently these oscillatory phenomena taking place in nerves, on the one hand, and those oscillatory functions of crystalline materials, on the other, are considered as analogous phenomena brought about by electron processes.

Summarizing what has been said in these lines: nerves — besides being information channels — are able to code the received information while transforming it by frequency modulation into a series of spikes, after which they propagate the information in this coded form.

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* Photoelectric phenomena are considered as being brought about by electron processes (e.g. Lakatos, 1966, 1967).

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Formulation of Some Problems of Cell Differentiation and Embryo Development on the Basis of the Theory of Self-Reproducing Automatons

(Theoretical paper)

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The process of cell reproduction, cell differentiation and some problems of regulations during embryo development are formulated on the basis of the theory of self-reproducing automatons. It has been shown that both in cell differentiation and in the regulation of embryo development besides the genetic information carried by the cells, the information provided by the mother and the information exchange between the different cells of an embryo play probably an important role.

Introduction

As it is well known, Neumann (1951) has shown on the basis of the considerations of Turing (1936), that if we have an automaton A which possesses a complexity greater, than a critical complexity, this automaton may reproduce itself. Namely if we provide to the automaton the suitable informations (programme) $\mathscr{I}$ and at the same time the building stones to the formation of the new automaton are also present, A can construct a new automaton which is identical with itself and contains the same information $\mathscr{I}$, as the original automaton,

$$A(\mathscr{I}) \rightarrow A(\mathscr{I}) \qquad \text{(self reproduction)}. \tag{1}$$

On the other hand if we give to the automaton A such an information $\mathscr{I}'$ which is not equal with $\mathscr{I}$, A may construct another automaton A', which may be also capable of self-reproduction and it may be also more complex, than A. Thus we can write

$$A(\mathscr{I}') = A'(\mathscr{I}'') \qquad \text{(construction of a new kind of automaton)},$$

where

$$\mathscr{I}'' = \mathscr{I}', \text{ or } \mathscr{I}'' \neq \mathscr{I}'. \tag{2}$$

Cell differentiation

It is known that in all cells of a higher organism the ensemble of DNA molecules contains the same genetic information I_G, but in the different kinds of cells a different part of the total information I_G is used to determine which protein should be synthetized in a given cell. If we denote these "used" informations in the different kinds of cells by

$$\mathcal{I}_{G_1}, \mathcal{I}_{G_2}, \mathcal{I}_{G_3}, \ldots, \mathcal{I}_{G_n},$$

we can write

$$\mathcal{I}_{G_1}, \mathcal{I}_{G_2}, \mathcal{I}_{G_3}, \ldots, \mathcal{I}_{G_n} \in I_G, \tag{3}$$

where n stands for the number of different kinds of cells. Histone molecules and other, only partially known regulation systems, will determine which part $\mathcal{I}_G$ of the total information I_G in a given kind of cell, should be transcribed to the RNA molecules and from it to be translated to the amino acid sequences of the synthetized proteins of the cells. Thus finally I_G determines which kind of cell should be constructed within the organism.

Let us consider the fertilized egg cell as an automaton A_1 and the used part ($\mathcal{I}_{G_1}$) of the total information of its DNA molecules as the programme necessary for the self-reproduction of this automaton. Then by analogy to (1) we can write

$$A_1(I_G|\mathcal{I}_{G_1}) \rightarrow A_1(I_G|\mathcal{I}_{G_1}) \quad \text{(self-reproduction of fertilized egg cell).} \tag{4}$$

In reality, however, the fertilized egg cell does not exist *in vitro*, but in the case of mammals in close contact with the mother. Therefore we have to take into account also the information $\mathcal{I}_{M_1}$ which comes from the mother and which may influence the self-reproduction of A_1. So it is more correct to say that the self-reproduction of A_1 occurs under the influence of both $\mathcal{I}_{G_1}$ and $\mathcal{I}_{M_1}$

$$A_1(I_G|\mathcal{I}_{G_1}) \xrightarrow{\mathcal{I}_{M_1}} A(I_G|\mathcal{I}_{G_1}). \tag{5}$$

If further self-reproductions have occurred there can be also an information exchange between the different fertilized egg cells. Let us designate by $\mathcal{I}_{A_1}; [A_1]$ the informations obtained by a given cell from all the other cells of the same kind. (The symbol $[A_1]$ denotes all the cells A_1 with the exception of the one under consideration.) In the language of the automaton theory we can then say that the reproduction of the automaton A_1 is controlled by the informations $\mathcal{I}_{G_1}, \mathcal{I}_{M_1}, \mathcal{I}_{A_1;[A_1]}$

$$A_1(I_G|\mathcal{I}_{G_1}) \xrightarrow{\mathcal{I}_{M_1}, \mathcal{I}_{A_1;[A_1]}} A_1(I_G|\mathcal{I}_{G_1}) \tag{6}$$

(self-reproduction of a fertilized egg cell in the presence of other cells of the same kind).

Since very probably both processes (5) and (6) take place[1], the information $\mathscr{I}_{A_1,[A_1]}$ must be such that it should not change the process of self-reproduction of A_1.

During one of the reproductions of A_1 it may occur that $\mathscr{I}_{M_1}$ changes to a different $\mathscr{I}'_{M_1}$ or $\mathscr{I}_{A_1,[A_1]}$ to a different $\mathscr{I}'_{A_1,[A_1]}$ and this may cause the reproduction of A_1 with a different $\mathscr{I}_{G_2}$ information[2, 3]

$$A_1(I_G|\mathscr{I}_{G_1}) \xrightarrow{\mathscr{I}_{M_1},\mathscr{I}'_{A_1;\,[A_1]}} A_1(I_G|\mathscr{I}_{G_2})$$

(change of available genetic information). (7)

Cell A_1 with this changed information $\mathscr{I}_{G_2}$ will then produce a different kind of cell A_2. Thus a cell differentiation:

$$A_1(I_G|\mathscr{I}_{G_2}) \xrightarrow{\mathscr{I}_{M_1},\mathscr{I}'_{A_1;\,[A_1]}} A_1(I_G|\mathscr{I}'_{G_2})$$

(cell differentiation), (8)

where $\mathscr{I}'_{G_2}$ can be equal or unequal with $\mathscr{I}_{G_2}$. The new kind of cell A_2 can then start to reproduce itself,

$$A_2(I_G|\mathscr{I}_{G_2}) \xrightarrow{\mathscr{I}_{M_2},\,\mathscr{I}_{A_2;[A_1]}} A_2(I_G|\mathscr{I}_{G_2} \xrightarrow{\mathscr{I}_{M_2},\,\mathscr{I}_{A_2;\,[A_1]},\mathscr{I}_{A_2;\,[A_2]}} A_2(I_G|\mathscr{I}_{G_2})$$

(self-reproduction of the differentiated cell) (9)

and so on. Here $\mathscr{I}_{M_2}$ denotes the information obtained from the mother by A_2 (generally $\mathscr{I}_{M_1} \neq \mathscr{I}_{M_2}$), $\mathscr{I}_{A_2;\,[A_1]}$ is the information obtained by A_2 from the ensemble of the A_1 cells and finally $\mathscr{I}_{A_2;\,A_2}$ stands for the informations obtained by A_2 from the other A_2 cell. If we have already a number of self-reproductions of A_2, we have to write instead of $\mathscr{I}_{A_2;\,A_2}$ again $\mathscr{I}_{A_2;\,[A_2]}$.[4]

[1] It seems rather probable that the fertilized egg cell first reproduces itself at least several times and cell differentiation occurs later only.

[2] The gross structure of cell A_1 remains the same, but its internal control system is changed in such a way that the used part of I_G changes from $\mathscr{I}_{G_1}$ to $\mathscr{I}_{G_2}$.

[3] It seems probable that with the increase of the amount of A_1 there is a change in $\mathscr{I}_{A_1,[A_1]}$. The direction of this change may be such that the change of $\mathscr{I}_{G_1}$ to $\mathscr{I}_{G_2}$ becomes more probable.

[4] It should be pointed out that the informations obtained by A_2 from the other A_2 and A_1 cells are not at all necessarily additive. Namely the information given to A_2 by a cell of kind A_1 may be strongly influenced by the presence of the other cells of kinds A_1 and A_2 and the same may hold also for the information given to A_2 by another A_2 cell.

In a similar way further differentiations of the cells A_1 and A_2 may occur,

$$A_2(I_G|\mathscr{T}_{G_2}) \xrightarrow{\mathfrak{J}_{M_2},\, \mathfrak{J}'_{A_2;[A_1,\,A_2]}} A_2(I_G|\mathscr{T}_{G_3}) \xrightarrow{\mathfrak{J}_{M_2},\, \mathfrak{J}_{A_2;\,[A_1,A_2]}} A_3(I_G|\mathscr{T}'_{G_3}) \tag{10}$$

$$A_1(I_G|\mathscr{T}_{G_1}) \xrightarrow{\mathfrak{J}_{M_1},\, \mathfrak{J}'_{A_1;\,[A_1,\,A_2]}} A_1(I_G|\mathscr{T}_{G_4}) \xrightarrow{\mathfrak{J}_{M_1},\, \mathfrak{J}_{A_1;\,[A_1,\,A_2]}} A_4(I_G|\mathscr{T}_{G_4}) \tag{11}$$

where we have used for the sake of brevity the notation

$$\mathscr{T}_{A_1;\,[A_1,\,A_2]} = \mathscr{T}_{A_1;\,[A_1]},\, \mathscr{T}_{A_1;\,[A_2]}\,. \tag{12}$$

Step (11) may occur if there are present only cells of kind A_1, but it may happen that it can occur only in the presence of both A_1 and A_2 cells or only in the presence of all three kinds of cells, A_1, A_2 and A_3. This situation can be expressed symbolically as follows:

$$A_1 \xrightarrow{\mathfrak{J}_{A_1;\,[A_1]}} A_4 \text{ or } A_1 \xrightarrow{\mathfrak{J}_{A_1;\,[A_1,\,A_2]}} A_4 \text{ or } A_1 \xrightarrow{\mathfrak{J}_{A_1;\,[A_1,\,A_2,\,A_3]}} A_4\,. \tag{13}$$

The new kinds of cells will again reproduce themselves, further cell differentiations may occur and so on. In this way a superautomaton $\mathscr{A}$, the organism itself will be formed which is capable of producing new superautomatons, other organisms,

$$\mathscr{A}(I_{G;\,[A_1,\,A_2,\,A_3,\,A_4,\ldots\ldots,\,A_n]}) \to \mathscr{A}'(I'_{G;\,[A'_1,\,A'_2,\,A'_3,\,A'_4,\ldots\ldots,\,A'_n]}). \tag{14}$$

where the primed quantities are not equal with the unprimed ones.

Embryo development

The question may be raised how the different kinds of automatons (cells) which are produced by cell differentiations, can form a superautomaton (organism). In other words we have to look for a regulation system which makes sure that only such kinds of cells will be reproduced which can cooperate with each other to form an organism (the information exchange between them is suitable for this purpose). To formulate this problem let us consider the i-th kind of cells A_i and let us denote by $A_{i_1}, A_{i_2}, \ldots, A_{i_k}, \ldots, A_{i_{n_i}}$ those cells of kinds n_i which can be formed from the cells of kind A_i in one differentiation step

$$A_i \to \begin{cases} A_{i_1} \\ A_{i_2} \\ \vdots \\ \vdots \\ A_{i_{n_i}} \end{cases} \tag{15}$$

(no other kind of cell A_k is formed before the members of the series of cells,

A_{i_1} $A_{i_2}, \ldots, A_{in_i}$ have been produced[5]). Steps (15) are assumed to be valid, of course, only in a given embryo (as we have seen before the differentiations of the cells A_i may be influenced by the informations $\mathcal{J}_{M_i}$ and $\mathcal{J}_{A_i\,[A_1;\,A_2,\,\ldots]}$ which are, of course, different in different embryos).

The newly formed kinds of cells, A_{i_1}, $A_{i_2}, \ldots, A_{in_i}$, can be divided into three groups. The first group comprises those cells A_i $(g = 1, 2, \ldots, k_i)$ which are capable of self-reproduction in the embryo and at the same time can cooperate with the other kinds of cells built up in the organism. To the second group belong cells A_{i_h} $(h = 1, 2, \ldots, l_i)$ which can reproduce themselves neither in the embryo, nor in a tissue culture (lethal cell differentiations) and therefore only a few of them are produced in the processes (15). There is a difficulty, however, with the cells A_{ij} $(j = i, 2, \ldots, m_i;\ k_i + l_i + m_i = n_i)$ which reproduce themselves in tissue cultures, but at the same time are not suitable for the cooperation with other kinds of cells to form the given organism.

To overcome this difficulty we may assume that those informations which, in addition to the genetic information $\mathcal{J}_{G_{ij}}$ of the non-cooperative cells A_{ij}, influence embryo development will inhibit the reproduction of these cells. This means that informations $\mathcal{J}_{M_{ij}}$ and $I_{A_{ij};\,[A_1,\,A_2,\,\ldots]}$ due to the mother and to the presence of the other kinds of cells make impossible the duplication of the non-cooperative cells A_{ij}.

$$A_{ij}\,(I_G/\mathcal{J}_{ij}) \xrightarrow{\mathcal{J}_{Mij},\ \mathcal{J}_{Aij},\ [A_1,\ A_2,\ \ldots]} A_{ij}\,(I_G/\mathcal{J}_{ij}) \tag{16}$$

In other words the total genetic information I_G of the fertilized egg cell and that of the mother I'_G, if taken together, are such that the ensemble of informations coming from the other cells to the cells A_{ij}, and the information coming to it form the mother,

$$I_G/\mathcal{J}_{G_1} \rightarrow \mathcal{J}_{A_{ij};\ [A_1,\ A_2,\ \ldots]} \tag{17}$$

$$\uparrow\ \mathcal{J}_{M_1}, \mathcal{J}_{M_2}, \ldots$$

$$I'_G \rightarrow \mathcal{A}_{\text{mother}}\,(I'_G;\,[A_{1m}, A_{2m}, \ldots]) \rightarrow \mathcal{J}_{M_j} \tag{18}$$

form *together* such a regulation system which inhibits the reproduction of the non-cooperative cells. It should be mentioned that I'_G determines also the informations $\mathcal{J}_{M_1}, \mathcal{J}_{M_2}, \ldots$ which can influence the informations $\mathcal{J}_{A_{ij};\,[A_1,\,A_2,\,\ldots]}$ (the vertical arrow between equations (17) and (18) is meant to indicate this).

We can conclude that if the assumed mechanism is correct, during evolution the total genetic informations I'_G, and I_G, respectively, have evolved in such a way as to exclude the reproduction of the non-cooperative differentiated cells.

[5] This definition, of course, does not exclude the possibility that several kinds of cells A_{i_k} may be produced in one step from different kinds of cells (for instance $A_{3_4} = A_{2_5}$; $A_3 \rightarrow A_{3_4}$, $A_2 \rightarrow A_{2_5}$).

This means, of course, restrictions on those possible total genetic informations I_G which may give rise to the development of an organism. At the same time the above outlined rather formal considerations and hypotheses may be useful in the cybernetical interpretation of the well-known species specificity of cross-breeding and of the species specificity of the mother in which fertilized egg cells of given specificity are implanted.

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Data on the α-Glycerophosphate Dehydrogenase-Substrate Complexes

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(Received December 5, 1967)

A pH dependent inhibition of α-glycerophosphate dehydrogenase activity by excess α-glycerophosphate is shown. The inhibition by excess substrate is explained by the existence of abortive complexes.

No abortive complex is formed with dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate.

The kinetic data as well as the effect of substrates on the heat stability and fluorescence of α-glycerophosphate dehydrogenase confirm the assumption of a compulsory order of ternary complex formation, with the coenzymes as first participants.

A study of the effect of substrate analogues shows that the binding group of α-glycerophosphate may be different from that of dihydroxyacetone phosphate or D-glyceraldehyde-3-phosphate. The latter two substrates are assumed to be bound through their phosphate groups.

Introduction

In a previous paper we have shown that GDH* catalyzes the reversible oxido-reduction between GAP and NADH (Telegdi, 1968). This reaction is similar to the reduction of DHAP which has previously been regarded to be the specific reaction catalyzed by GDH (Baranowski, 1949). GDH catalyzes the following parallel reactions, the relative rate of which is pH-dependent (Telegdi, 1968):

$$\text{DHAP} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{GP} + \text{NAD}^+$$
$$\text{GAP} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{GP} + \text{NAD}^+$$

It was of great interest to study the enzyme-substrate binding with a special care for the two different pathways described above.

* Abbreviations used: GDH: α-glycerophosphate dehydrogenase (L-glycerol-3-phosphate: NAD oxidoreductase, E.C.1.1.1.8); GP: α-glycerophosphate; GAP: D-glyceraldehyde-3-phosphate; DHAP: dihydroxyacetone phosphate; NAD and NADH: oxidized and reduced nicotinamide-adenine-dinucleotide, respectively; NADP: nicotinamide-adenine-dinucleotide phosphate, oxidized; AMP: adenosine monophosphate; FDP: fructose-1,6-diphosphate.

There are only a few recent data concerning the mechanism of formation of enzyme-substrate complexes and the reaction of GDH. Black (1966) found in kinetic studies the inhibition of the enzyme by an excess of DHAP, NADH, or GP. An ordered mechanism of the formation of ternary complexes was demonstrated in kinetic studies (Black, 1966) and various kinds of inactive complexes were suggested to be responsible for substrate inhibition (Black, 1966; Apitz et al., 1964). Apitz et al. (1964) assumed a random, "general" mechanism in the formation of ternary enzyme-substrate complexes with GDH.

The intention of the present work was to obtain data about the conditions for the formation of enzyme-substrate complexes. For this purpose the pH-dependence of the kinetic constants and the effect of an excess of substrates and substrate analogues on the enzyme were investigated.

Materials and Methods

GDH was isolated from rabbit skeletal muscle and purified as described earlier (Telegdi, 1964). A molecular weight of 78 000 was used in the calculations.

A Rhône-Poulenc preparation of GP was used. GAP was prepared according to Szewczuk et al. (1961) from fructose-1,6-diphosphate (Reanal). DHAP was prepared from GAP (Telegdi, 1968). 85 per cent pure NAD (Boehringer) was used and 75–80 per cent pure NADH (Reanal). The acetylphosphate (dilithium, monoacetyl salt) was a preparation of Light and Co. Erythrose-4-phosphate was a gift of Dr. G. Dénes (Institute of Medical Chemistry, University Medical School, Budapest).

Enzymic activity was measured by Warburg's optical test in a Hilger UVISPEK spectrophotometer as described earlier (Telegdi, 1964).

Results

The influence of pH on the kinetic constants

The substrate saturation of GDH with GP is shown in Fig. 1.

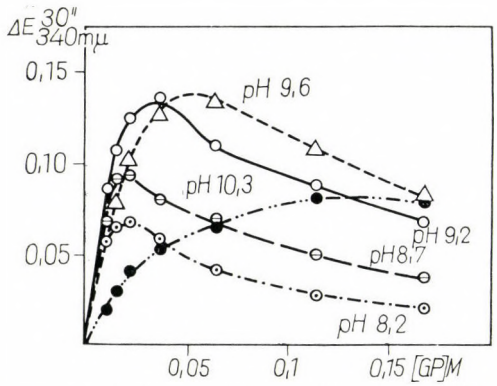

Fig. 1. Saturation of GDH with GP at different pH values. GDH: 6.4×10^{-6} μmole/ml; NAD: 2.0×10^{-4} M. In 0.1 M glycine buffer

As seen in Fig. 1, the oxidation of GP is inhibited by an excess of this substrate. The inhibitory effect is pH-dependent. A marked inhibition can be observed below the optimal pH (pH 9.6). Above the pH optimum there is no inhibition with an excess of substrate.

The Lineweaver-Burk plot of saturation with GP at different pH values is shown in Fig. 2.

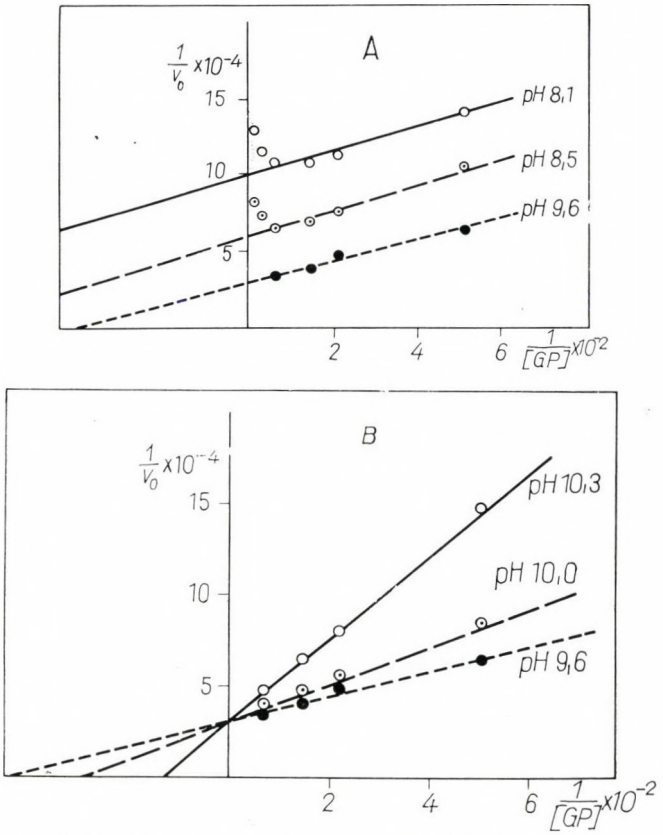

Fig. 2. Lineweaver-Burk plot of the saturation of GDH with GP. A: At and below the pH optimum. B: At and above the pH optimum. Conditions see in legend to Fig. 1

Two different types of inhibition are indicated by the parallel lines obtained below the pH-optimum and by the competitive pattern obtained above the pH-optimum, respectively. At the same time, the K_M value of GP shows a monotonous increase as the pH is increased from pH 8.1 to 10.3.

Both the low K_M values and the inhibition with the excess of substrate below the pH optimum are suggested to be due to the formation of "abortive complexes".

The K_M of substrates at different pH values is shown in Table 1.

Table 1

K_M (in M) and V_{max} values of various substrates at different pH values
NADH oxidation was measured in 0.1 M tris buffer, NAD reduction in 0.1 M glycine buffer, with 3.8×10^{-6} μmole/ml GDH

pH	GP+		DHAP++		GAP§		NAD§§		NADH*,□	
	$K_M \times 10^3$	$V_{max} \times 10^5$	$K_M \times 10^4$	$V_{max} \times 10^5$	$K_M \times 10^4$	$V_{max} \times 10^5$	$K_M \times 10^4$	$V_{max} \times 10^5$	$K_M \times 10^5$	$V_{max} \times 10^5$
7.5	—	—	—	—	1.6	0.77	—	—	3.0	0.9
8.0	0.57	1.0	2.5	3.3	4.3	0.77	—	—	2.2	0.8
8.5	0.66	1.6	5.0	3.3	6.6	0.77	0.52	1.6	1.5	0.7
8.9	—	—	10.0	3.3	—	—	—	—	—	—
9.6	2.2	3.3	—	—	—	—	1.1	4.0	—	—
10.0	3.3	3.3	—	—	—	—	—	—	—	—
10.3	6.6	3.3	—	—	—	—	2.0	4.0	—	—

* Measured with GAP as substrate.
\+ NAD: 1.9 μmole/ml
++ NADH: 1.9×10^{-1} μmole/ml
§ NADH: 1.9×10^{-1} μmole/ml
§§ GP: 5.8 μmole/ml
□ GAP: 1.9 μmole/ml

It can be seen that the K_M of NAD and NADH are lower by one order of magnitude than that of the corresponding substrates. An ordered mechanism of ternary complex formation, where the binding of coenzyme is the first step, which was suggested by Black (1966), is in agreement with these data.

The following reactions are assumed to take place:

$$GP + E + NAD \rightleftharpoons GP + E\text{-}NAD \rightleftharpoons GP\text{-}E\text{-}NAD \rightleftharpoons DHAP(GAP) - E\text{-}NADH$$
$$DHAP(GAP) - E\text{-}NADH + GP \rightleftharpoons GP\text{-}E\text{-}NADH \text{ (inactive)} + DHAP(GAP)$$

If GP is bound to the binding site of DHAP or GAP, a GP-E-NADH complex is formed. This complex is inactive if the dissociation of NADH is inhibited by the bound GP.

The idea is supported by the competition between GAP and GP for the binding site of GAP at pH 8.5 (Fig. 3).

This competition indicates that below the pH optimum GP is bound to the E-NADH complex at the binding site of GAP. A similar competition between GP and DHAP was demonstrated by Blanchaer (1965).

The pH dependence of both the K_M values and the inhibition by GP can be explained by the effect of pH on the formation of the abortive complex. Such complexes can only be formed below the pH optimum. As the pH is increased the significance of inactive complexes is decreased. Under these conditions both K_M and V_{max} are increasing. Above the pH optimum a further increase of K_M can be observed, while V_{max} becomes constant. The absence of substrate inhibition indicates that no abortive complex is formed at high pH values.

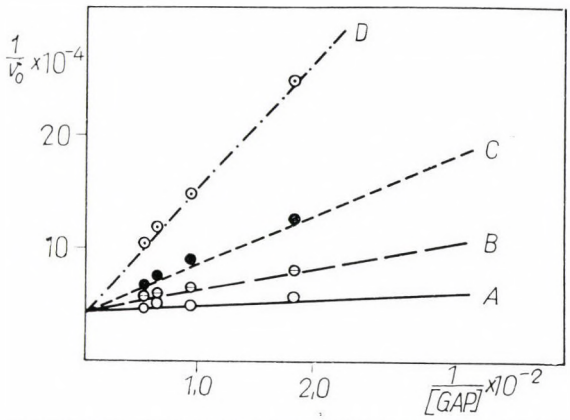

Fig. 3. Lineweaver-Burk plot of the inhibition of GAP reduction by GP. GDH: 1.9×10^{-5} mole/ml; NADH: 1.76×10^{-1} μmole/ml. In 0.1 M tris buffer, pH 8.5. A: without GP; B: 1.0×10^{-2} M GP; C: 2.0×10^{-2} M GP; D: 3.0×10^{-2} M GP

An excess of the other substrates of GDH has no inhibitory effect under our experimental conditions. The Lineweaver-Burk plots of saturation by DHAP and GAP at different pH values show a competition between $[H^+]$ and these substrates, but no inhibition by an excess of DHAP or GAP.

The effect of substrates on the stability and structure of GDH

The influence of substrates and coenzymes on the thermal inactivation of GDH was measured at 40° C and 55° C, in 0.1 M glycine buffer, pH 6.5 and 8.5. The loss of enzymic activity was followed by determining the rate of oxidation of GP (Table 2).

Table 2

The effect of substrates and coenzymes on the heat stability of GDH
GDH: 20 μg/ml; GP or GAP: 3 mg/ml; NAD or NADH: 2 mg/ml. Preincubation in 0.1 M tris buffer, pH 6.5. The same results were obtained in pH 8.5 buffer

Sample tested	Per cent of original activity					
	incubated at 20°C		40°C		55°C	
	for 2′	20′	2′	20′	2′	20′
GDH	100*	100	100	98	15	6
GDH + GP	100	98	98	93	20	12
GDH + GAP	100	96	100	95	5	0
GDH + NAD	100	88	8	5	7	7
GDH + NADH	100	100	97	96	100	94

* Initial activity is taken for 100. Other data refer to this arbitrary value.

The opposite effect of NADH and NAD is remarkable. While the enzyme is markedly stabilized in the presence of NADH, the stability of the enzyme is strikingly decreased by NAD. GP and GAP are ineffective under the same conditions. Differences in the effect of substrates on the protein structure were assumed to be responsible for their different effects on the stability. Therefore, the fluorescence spectrum of the enzyme was studied in the presence of substrates.

The value of maximum fluorescence at 340 mμ depends on the number of ionizable tyrosine and tryptophan residues excited at 290 mμ (Shore, Pardee, 1956). In the presence of NADH the value of this maximum is considerably decreased (Fig. 4).

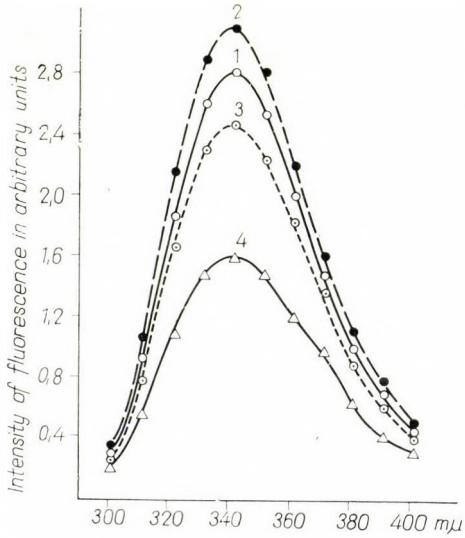

Fig. 4. Fluorescence spectrum of GDH excited at 290 mμ. GDH: 50 μg/ml in 0.1 M glycine buffer, pH 8.5. 1: GDH alone; 2: +2 mole equivalents of NAD or DHAP; 3: +2 mole equivalents of GP or GAP; 4: +2 mole equivalents of NADH

This decrease of the fluorescence may be ascribed to the alteration of the steric structure of the protein molecule (Joly, 1965) induced by NADH, that is, to the formation of more compact molecules having a much higher stability as compared to the native enzyme. NAD or the substrates have only a slight effect on this spectrum. Accordingly, the mechanism of the loss of stability due to the binding of NAD cannot be simply explained by an induced loosening of the protein structure.

A considerable increase in fluorescence was observed at 460 mμ (excited at 380 mμ, see Keleti, 1968) upon the addition of NAD or NADH to the enzyme (Fig. 5).

This fluorescence of GDH is not altered in the presence of substrates.

From the increasing intensity of the fluorescence of GDH in the presence of NAD, the number of coenzyme molecules bound to the enzyme could be

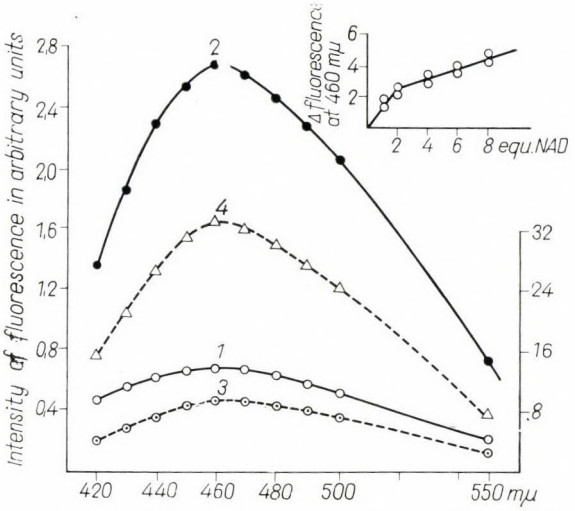

Fig. 5. Fluorescence spectrum of GDH excited at 380 mμ. GDH: 1.002 mg/ml in 0.1 M glycine buffer, pH 8.5. 1: GDH alone or GDH + 2 mole equivalents of GP or GAP or DHAP; 2: GDH + 2 mole equivalents of NAD; 3: NADH alone; 4: NADH + 0.5 mole equivalent of GDH (in this case = 1.002 mg/ml). The left-hand ordinate refers to the continuous lines, the right-hand ordinate refers to the dotted lines

determined (Fig. 5). As seen in Fig. 5, the binding of 2 moles of NAD per mole of GDH has been measured.

The effect of substrate analogues on the activity of GDH

The effect of various substrate analogues on the activity of GDH was studied. A difference was observed in the effect of inorganic phosphate on the rate of the forward and the reverse reaction.

Phosphate ions have a strong inhibitory effect on the reduction of DHAP (Nagradova, 1959) and GAP. A competition has been found between these substrates and the phosphate ions (Fig. 6).

It is remarkable, that the oxidation of GP is not inhibited by phosphate ions. On the contrary, a slight activation occurs in the presence of phosphate.

The competition between inorganic phosphate and the triose phosphates shows that these substrates may be bound to the enzyme through their phosphate groups. The binding of GP is presumably different.

In Table 3 the influence of different substrate analogues and of o-phenanthroline on the activity is summarized.

It is seen that acetyl phosphate inhibits the activity of both the forward and the reverse reactions. In the case of reduction of DHAP and GAP, the inhibition may be due to the phosphate group of acetyl phosphate.

The oxidation of GP is inhibited by acetyl phosphate, erythrose-4-phosphate and o-phenanthroline, but the other compounds studied have no effect on this reaction.

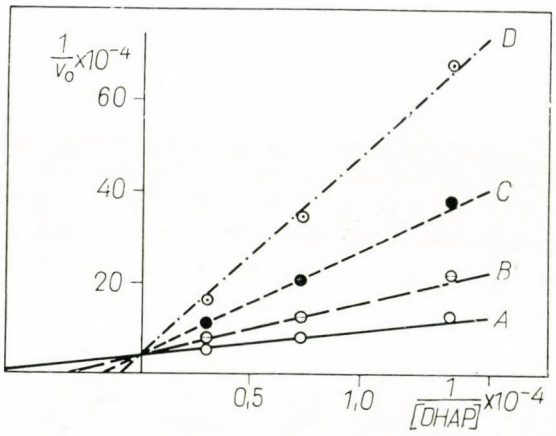

Fig. 6. Lineweaver-Burk plot of the inhibition by phosphate ions of DHAP reduction. GDH: 7.6×10^{-6} μmole/ml in 0.1 M tris buffer, pH 8.5; NADH: 1.76×10^{-1} μmole/ml. A: without phosphate; added Na_2HPO_4 in M, B: 0.016; C: 0.048; D: 0.08

Table 3

The effect of substrate analogues and other compounds on the activity of GDH

Compound tested	Oxidation of GP	Reduction of DHAP or GAP
	100*	100*
Na_2HPO_4, 4.8×10^{-2} M	112	42
Glycerin, 3×10^{-1} M	97	101
D-glyceraldehyde, 1.5×10^{-1} M	105	98
L-lactate, 2.5×10^{-2} M	94	96
Acetyl phosphate, 1.4×10^{-2} M	57	48
Erythrose-4-phosphate, 5×10^{-3} M	63	67
FDP, 8×10^{-3} M	92	98
Pyrophosphate, 1.6×10^{-2} M	105	100
1,10-phenanthroline, 1.5×10^{-3} M	40	100

* Control activity is taken for 100. Results are given in the percentage of control activity.

The binding of GP to the enzyme through its phosphate group is excluded, since no inhibition of GP oxidation has been obtained by phosphate ions. A reasonable assumption is the binding of GP to the enzyme at the phosphate ester bond. This is the only group which is present in GP as well as in the two analogous compounds mentioned above, while absent in the others which do not inhibit the oxidation of GP.

The other compounds studied have no effect on the rate of the reactions catalyzed by GDH either in the forward or in the reverse direction.

Discussion

The K_M values of NAD and NADH in the presence of substrates are much lower, than those of the substrates in the presence of coenzyme. The binding of the coenzyme to the enzyme in the ternary complex may be more stable than the binding of substrate in the same complex.

The heat stability of GDH is strongly dependent on the presence of coenzyme, whereas the substrates are ineffective in the absence of coenzyme.

As shown by the effect of NAD and NADH on the fluorescence of GDH, they may cause structural changes in the enzyme. The substrates have no such effect.

These data are in good agreement with the results of Black (1966) who assumed from kinetic data a compulsory order of ternary complex formation where the first step is the binding of coenzyme to the free enzyme.

Differences were found in the effect of excess of different substrates on the enzymic activity. While the excess of DHAP and GAP had no inhibitory effect, a strong inhibition was found with the excess of GP. Inhibition by a high concentration of GP was observed at the pH 9.6 optimum and at lower pH values, but no inhibition could be detected above the optimum. This phenomenon is explained by assuming the formation of an NADH-E-GP abortive complex below the pH optimum. When an NADH-E-GP complex is formed this complex is inactive, if GP inhibits the dissociation of NADH from the ternary complex.

DHAP and GAP may be bound to the enzyme through their phosphate group since phosphate inhibits competitively the reduction of DHAP and GAP. In the active GP-enzyme complex GP may be bound through some other group since phosphate has no effect on GP oxidation. However, even GP may be bound to the enzyme through the phosphate group, under certain conditions. This can be the case below the pH optimum, with GP being bound to the binding site of the phosphate group of DHAP or GAP on the enzyme, thus yielding an abortive complex.

Our experiments with various substrate analogues are in agreement with data showing no effect of pyrophosphate on the oxidation of GP or reduction of DHAP (Nagradova, 1959; Ankel et al., 1960) and also with the observed inhibition of the reduction of DHAP by acetyl phosphate (van Eys et al., 1958).

The phosphate ester bond may be involved in the binding of GP in the active GP-enzyme complex, since only acetyl phosphate and erythrose-4-phosphate inhibit the oxidation of GP. Some further data on the role of phosphate ester bond in the binding of substrate are furnished by Apitz et al. (1964a, 1965) who have shown a competition of NADP, 2'-AMP, 3'-AMP and other phosphate esters with the substrate.

As for the groups of the enzyme involved in the binding of substrates, it is known that during the reaction catalyzed by GDH an intermediate product is formed (Telegdi, 1968) which may be bound to the reactive SH group of the enzyme (Sajgó, Telegdi, 1968) not involved in the binding of the original substrate (Telegdi, Keleti, 1966). The groups of the enzyme which binds GP may be different from that which binds GAP or DHAP, since o-phenanthroline inhibits

the oxidation of GP but has no effect on the reduction of GAP or DHAP. Consequently, it may be assumed that different groups (reacting with o-phenanthroline) are responsible for the binding of the reduced substrate, of the intermediate (probably the reactive SH group), and of the oxidized substrates.

Contrary to Black's data (Black, 1966) no inhibition has been found by an excess of DHAP, accordingly, no abortive complex formation involving DHAP can be assumed. Similar results have been obtained with GAP. In these cases the maximum velocity measured at the pH optimum is the same as those below and above the optimum.

Acknowledgements

The very valuable technical assistance of Miss I. Bak and Mrs. M. Szegvári is gratefully acknowledged.

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Study of the Physical Chemical and Enzyme Chemical Properties of Contractile Proteins in Postnatal Life

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Myosin and actomyosin fractions were prepared from the muscles of rabbits of different ages after the extraction of sarcoplasmatic proteins by the method given in the text. It was found that

1) In the first 24 days of postnatal life the specific activity of sarcoplasmatic cholinesterase remained practically unchanged (64 to 86 μM Ach per mg protein per hour), while in the muscles of fully developed animals the specific activity was about 15—20 per cent of the above value. The specific activity of myosin cholinesterase gradually decreased with the progress of postnatal life.

2) Components with sedimentation coefficients of 2.5—3.0 S, 6.5—7.5 S and 11.0—12.0 S were detected in the myosin fraction of animals in the first 24 days of their postnatal life, while 2.5—3.0 S, 6.5—7.5 S and 18.0—24.0 S components were found in the actomyosin fraction of the same animals. In the myosin and actomyosin fractions of fully developed animals the 2.5 S and the 11.0—12.0 S components were missing.

3) Both the myosin and the actomyosin fraction contained nucleic acid in the first weeks of postnatal life. In the myosin and actomyosin fractions of fully developed animals no nucleic acid or nucleotide was detected.

A possible correlation is suggested between myosin cholinesterase and the components with low sedimentation coefficients (2.5—7.5 S).

Introduction

In certain mammals all the limb muscles have a tonic function in the first stage of postnatal life, but these undergo during development a differentiation into rapid, tetanic and slow tonic muscles (Denny-Brown, 1929; Koshtoiants, Ryabinovskaya, 1935; Buller et al., 1960a, b). During ontogenic development the properties of muscles will change including the structure of the sarcoplasmatic reticulum of the muscle (Veratti, 1961; Ezerman, Ishikawa, 1967), the length of the sarcomera, the ion composition (Drahota, 1960, 1961; Mori, Duruisseau, 1960), as well as the adenosine triphosphatase activity (Hermann et al., 1949; Robinson, 1952; De Villafranca, 1954) and cholinesterase activity (Varga et al., 1957) of myosin.

This explains the recent interest in a comparison of the physical chemical and enzymological properties of contractile proteins obtained from tonic and tetanic skeletal muscle (Bárány et al., 1964, 1965a; Gergely et al., 1965) and in the

investigation of their ontogenic changes during ontogenesis (Pinaev, 1965; Bárány et al., 1965b; Ohshima et al., 1965).

In the present paper we shall attempt to describe the ontogenetic changes which occur in the physical chemical and enzymatic properties of the contractile protein fractions prepared from the striated muscle of the rabbit.

Part of these experiments have been reported at the 1966 Congress of the Hungarian Physiological Society (Szabolcs et al., 1966).

Methods

The limb muscles of rabbits of the same age and from the same litter were collected in frozen physiological saline. The cooled muscle minced with a pre-cooled mincing machine, then it was extracted two times by continuous stirring at 0° C for 15 minutes with 3 volumes per gram muscle of deionized water. Both supernatants were obtained by centrifugation of the homogenate 0 to +2° C in a refrigerated centrifuge. After extraction with water the preparation was twice extracted with 3 volumes of cold 0.05 M KCl solution per gram muscle. The aqueous and the 0.05 M KCl supernatants were pooled and stored in the cold for the determination of cholinesterase activity and protein content (for the extracted quantity of protein and for cholinesterase activity at low ionic strength, see Table 1).

Following the treatments mentioned above muscle pulp was extracted with 3 volumes of 0.3 M KCl + 0.15 M phosphate buffer at pH 6.5 for 30 minutes, and then centrifuged in a refrigerated centrifuge. Samples were taken for the determination of protein concentration and cholinesterase activity (Table 1, extracted at 0.6 μ). The sediment was discarded and the ionic strength of the supernatant was reduced to about 0.02 μ (with 14 volumes of cold deionized water). The protein precipitated at low ionic strength was collected by centrifugation. The precipitate was dissolved at 0.4 μ in a solution which was obtained by a 2/3 dilution of the 0.6 μ extracting solution containing 0.3 M KCl and 0.15 M phosphate. Samples were taken for the determination of protein and cholinesterase activity (Table 2), and the ionic strength was then reduced to 0.28 μ for removing actomyosin. The actomyosin which precipitated during incubation for 2–3 hours at 0° C was collected by centrifugation and then dissolved at 0.6 μ. The preparation obtained by this procedure was used in the experiments as actomyosin (Table 3). Myosin was prepared from the 0.28 μ supernatant by reducing the ionic strength to 0.05 μ (Table 3). This procedure follows that of Maercaud-Raeber et al. (1959).

The cholinesterase activity of the protein fractions of sarcoplasmatic and myofibrillar origin was measured with the method of Hestrin (1949) as follows:

The reaction mixture contained 0.05 M KCl, 1.5×10^{-3} M acetylcholine chloride (Hoffmann–La Roche, Basel) and 0.01 M TRIS-hydrochloride buffer, pH 8. The incubations were carried out for 30 and 60 minutes at 37° C.

The homogeneity of the preparations was examined by means of a PHYWE U-77 type ultracentrifuge. The records were taken with a Philpot-Svensson optical system. The percentage of components in the preparations under investi-

gation was determined by means of a MOM planimeter. In the calculation of the data in Table 3 the correction due to the Johnston-Ogston (1946) effect was neglected.

The optical absorption of the myosin and actomyosin fractions was measured with a Unicam SP 500 spectrophotometer.

Protein was determined by the micro-Kjeldahl method.

Results

In the first part of our experiments we determined the protein content of and the quantity of acetylcholine hydrolyzed by fractions extracted at low (up to 0.05 μ) and high (up to 0.6 μ) ionic strength from the muscle of rabbits of different ages.

It appears from Table 1 that the protein content and acetylcholine splitting of fractions obtained from the muscle at low ionic strength increases for some time with the age of the rabbits so that their specific cholinesterase activity, expressed in μM Ach per mg of protein per hour, maintains an approximately

Table 1

The quantity and cholinesterase activity of proteins extracted at low (0.0—0.05 μ) and high (0.60 μ) ionic strength from 100 g of rabbit muscle obtained from animals of different ages

Postnatal age (days)	Extracted at low μ			Extracted at high μ		
	g protein/100 g muscle	μM ACh decomposed/100 g muscle	Specific ChE activity	g protein/100 g muscle	μM ACh decomposed/100 g muscle	Specific ChE activity
5	2.871	2472.5	0.86	0.7578	1868	2.465
5	3.007	1925.0	0.64	0.890	1500	1.685
19	3.851	3022.5	0.785	0.878	1238	1.41
24	3.588	2775.5	0.77	1.013	1317	1.3
adult	6.755	870.0	0.1285	0.735	935	0.21

constant value. The protein content of fractions obtained by extraction of muscle pulp at high (0.6 μ) ionic strength following extraction at low ionic strength slightly increases with the age of the animals, while the rate of acetylcholine hydrolysis decreases. Consequently a decrease of specific activity can be observed.

Table 2 shows the total protein yield, the quantity of acetylcholine hydrolized by and the specific activity of the fractions containing both myosin and actomyosin which are obtained by extraction at 0.6 μ followed by precipitation at 0.02 μ and re-dissolution at 0.4 μ (see Methods).

A comparison of the data in Tables 1 and 2 shows that the reduction in the protein yield following precipitation at 0.04 μ is lower in the case of adult animals (about 43 per cent) than in young animals (about 65—80 per cent), indicating that the precipitability at 0.04 μ of proteins extracted at 0.6 μ changes

Table 2

The quantity and cholinesterase activity of proteins extracted at 0.60 μ ionic strength from 100 g of rabbit muscle as a function of the age of the animals

The extract was further purified by a single precipitation at 0.04 μ

Postnatal age (days)	g protein/100 g muscle	μM ACh decomposed/100 g muscle	Specific ChE activity
5	0.171	559.5	3.275
5	0.173	503.5	2.925
19	0.328	298.0	0.915
24	0.270	333.0	1.235
adult	0.416	75.05	0.18

in the course of postnatal life. This would also explain observed changes in acetylcholine hydrolysis and in specific activity.

Next we tried to clarify the basis of the reduction in the specific activity of cholinesterase and, therefore, we investigated the ultracentrifugal homogeneity of myosin and actomyosin fractions prepared from the muscle of rabbits of different ages.

Figure 1 shows the homogeneity of myosin prepared from 5 days old and adult rabbits, and the homogeneity of actomyosin prepared from 24 days old rabbits.

It appears from Fig. 1 that the myosin prepared from 5 days old rabbits contains three components (Fig. 1b) with the following sedimentation coefficients: 2.7 S, 7.5 S and 11.5 S. The myosin prepared from the muscle of adult rabbits is homogeneous (Fig. 1a), with a sedimentation coefficient of 4.6 S at 6 mg per ml protein concentration. The actomyosin fraction prepared from the muscle of 24 days old rabbits contains three components (Fig. 1c) with sedimentation coefficients of 2.65 S, 6.4 S and 18.6 S.

In view of the findings shown in Fig. 1 it appeared necessary to investigate a number of further myosin and actomyosin preparations from the muscle of rabbits of different ages. The results of these experiments are summed up in Table 3.

It is seen from Table 3, that with the exception of preparations obtained from the muscle of adult rabbits, both the myosin and the actomyosin fractions always contain components with a sedimentation coefficient of 2.5–3.0 S though in a varying percentage. The component with the sedimentation coefficient characteristic of myosin (6.5–7.5 S) is always present in the myosin fraction, and from about the 24th day of postnatal life this component will also appear in the actomyosin fraction. It can be further seen from Table 3 that the specific activity of cholinesterase in the actomyosin fraction decreases with the progress of postnatal life.

It appears that in the 4th and 5th day of postnatal life the cholinesterase activity of the myosin fraction is at least partially dependent on the presence

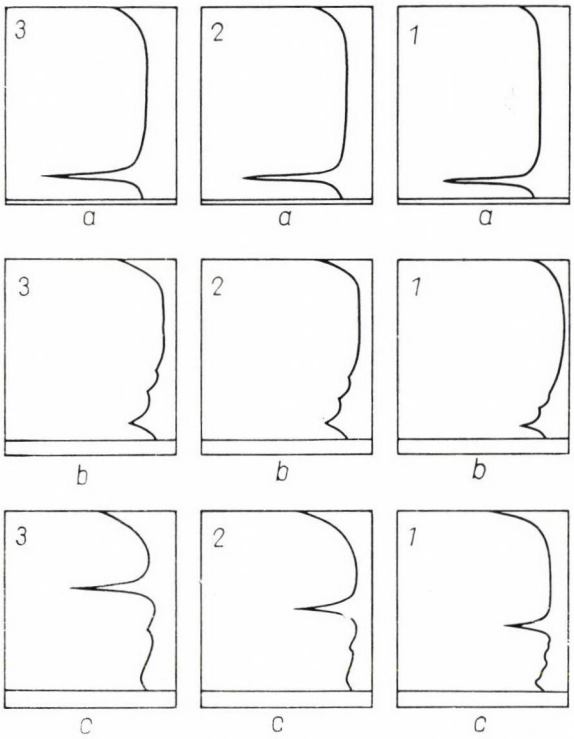

Fig. 1. Sedimentation pattern of myosin and actomyosin fractions prepared from the muscle of rabbits of different ages. *a)* homogeneity of the myosin fraction of adult rabbit muscle. Protein concentration 6 mg/ml; pH 7; 45 000 rpm. Philpot angle 20—25°; $t = 22.5°$ C. The first photographs were taken 30 minutes after acceleration, the others at 10-minutes intervals (from right to left). *b)* homogeneity of the myosin fraction of the muscle of five days old rabbits. Protein concentration 3.0 mg/ml; pH 7; 43 000 rpm. Philpot angle 50—55°; $t = 21.0°$ C. The first photograph was taken 30 minutes after acceleration, the others at 10-minutes intervals (from right to left). *c)* homogeneity of the actomyosin fraction of the muscle of 24 days old rabbits. Protein concentration 3.86 mg/ml; pH 7; 43 000 rpm. Philpot angle 30—40°; $t = 20°$ C. The first photograph was taken 20 minutes after acceleration, the others at 10-minutes intervals (from right to left).

of the 11.0—12.0 S component in the preparation. When this component is presen-cholinesterase activity will be lower. Cholinesterase activity at this stage of postt natal life is apparently determined by the amount of the components with a low sedimentation coefficient (2.5—7.5 S). A further elaboration of this idea is presented in the discussion.

Figure 2 shows the postnatal changes in the sedimentation coefficients of the components of the myosin fraction.

It is seen that in the first 24 days of postnatal life there is no significant change in the sedimentation coefficient of any of the three components of the myosin fraction.

The following experiments were carried out to prove that the cholinesterase activity of the actomyosin fraction is mainly due to a contamination by some

Table 3

Cholinesterase activity of myosin and actomyosin fractions prepared from the muscle of rabbits of different ages, and the percentage distribution of these fractions into ultracentrifugal components

Post-natal age (days)	Myosin fraction			Actomyosin fraction			Myosin fraction	Actomyosin fraction
	Sedimentation coefficient (S)						Cholinesterase activity	
	2.5–3.0	6.5–7.5	11–12	2.5–3.0	6.5–7.5	18–24	μM ACh/mg protein/hour	
	percentage of the total							
4	54.4	31.10	14.50	—	—	—	4.24	2.80
5	46.2	44.10	9.70	43.30	0	56.7	5.75	2.95
5	54.83	37.42	7.75	—	—	—	4.82	—
5	58.0	42.0	0	35.90	0	64.1	6.165	2.45
6	89.1	10.9	0	—	—	—	5.65	1.35
8	38.0	36.0	26.0	—	—	—	—	—
10	47.4	35.60	17.0	—	—	—	3.255	—
19	70.0	30.0	0	32.3	0	67.7	1.975	0.720
24	39.9	37.1	23.0	23.3	10.7	66.0	2.075	1.22
adult	0	100	0	0	10.0	90.0	0.0835	0.06

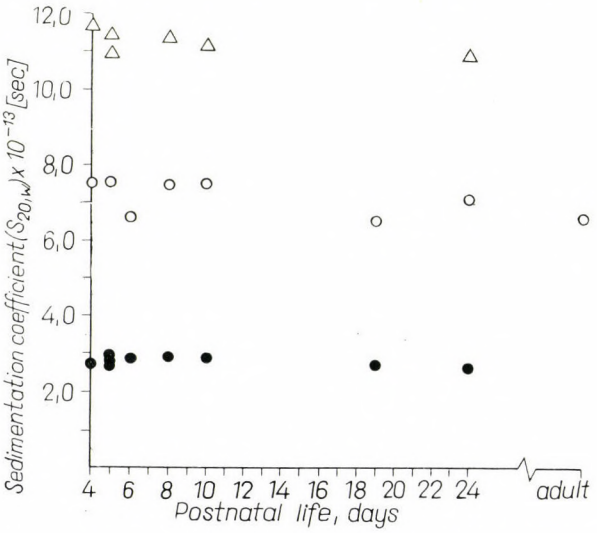

Fig. 2. Postnatal changes in the sedimentation coefficients of the components of the myosin fraction. ● — ●: 2.5—3.0 S component. ○ — ○: 6.5—7.5 S component. △ — △: 11.0—12.0 S component

components of the myosin fraction: myosin and actomyosin fractions were prepared from the muscle of 11 days old rabbits with the method described in the Methods, with the exception that the separation of myosin and actomyosin was done at different ionic strengths (0.3 μ; 0.25 μ and 0.20 μ). The distribution of cholinesterase activity between the myosin and actomyosin fractions obtained

at these ionic strengths (0.3 μ; 0.25 μ and 0.2 μ, respectively) was consequently determined.

It appears from Fig. 3 that the total cholinesterase activity of the myosin fraction decreases with decreasing ionic strength, while that of the actomyosin fraction increases. This indicates that as the solubility of myosin cholinesterase decreases with decreasing ionic strength it will be left in the actomyosin fraction.

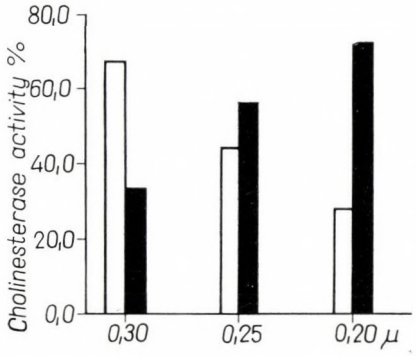

Fig. 3. The effect of ionic strength on the percentage distribution of cholinesterase activity between the myosin and actomyosin fractions. □ cholinesterase activity of the myosin fraction. ■ cholinesterase activity of the actomyosin fraction

In the second part of our experiments we investigated the spectrophotometric properties of the myosin and actomyosin fractions for a further physical chemical characterization of these preparations.

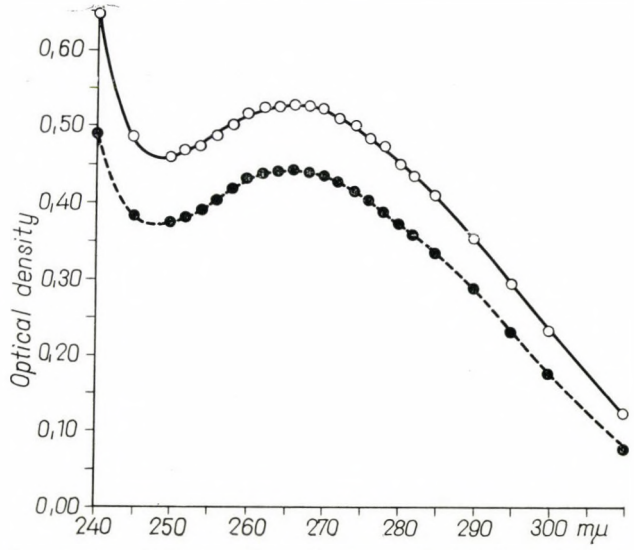

Fig. 4. Absorption spectra of the myosin and actomyosin fractions of the muscle of 5 days old rabbits. ● — ●: myosin fraction; protein concentration 0.124 mg/ml. ○ — ○ : actomyosinfraction; protein concentration 0.138 mg/ml

Figure 4 shows the absorption spectra of the myosin and actomyosin fractions which have been prepared from the muscle of five days old rabbits. The absorption maxima of both fractions are at 266 mμ, and this as well as the ratio of optical densities at 280 and 260 mμ (myosin fraction: 280/260 = 0.87, actomyosin fraction: 280/260 = 0.884) indicates the presence of bound nucleotide or nucleic acid in the preparations.

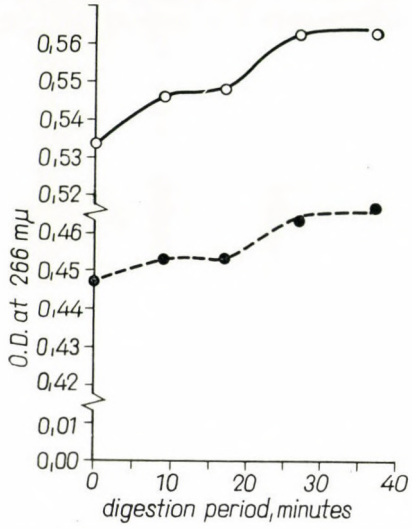

Fig. 5. Ribonuclease digestion of the myosin and actomyosin fractions of the muscle of 5 days old rabbits. ● — ●: myosin fraction; protein : ribonuclease = 5 : 1. ○ — ○: actomyosin fraction; protein : ribonuclease = 5.5 : 1

When the myosin and actomyosin fractions of 5 days old rabbits were digested with ribonuclease, the optical density of these preparations was increased.

The absorption maxima of the myosin and actomyosin fractions from the muscle of adult rabbits were at 278 mμ. The presence of nucleic acid could not be demonstrated; the ratio of the optical densities at 280 and 260 mμ was 1.6 and digestion with ribonuclease did not lead to an increase in absorbance.

Discussion

Varga et al. (1957) have shown that in the first days of postnatal life the cholinesterase activity (acetyl- + myosin cholinesterase) of rabbit muscle is about six to seven times higher than in adult animal. There is a considerable difference between our method of preparation – especially in the extraction – and that applied by Varga et al. nevertheless, our data in Table 1 also show that in the first weeks of postnatal life cholinesterase activity (cholinesterase extracted at low and high ionic strengths) is four to five times higher than that of the adult rabbit muscle. The difference in the cholinesterase activity per gram muscle

figures is most pronounced in the so-called total myosin cholinesterase activity which might be explained by the short (30 minutes) single extraction of the muscle pulp residue after the removal of the sarcoplasmatic proteins, and of acetylcholinesterase, respectively. The acetylcholinesterase quantities obtained from the muscle of adult animals (Table 1) are in good agreement with the data of Varga et al. (1957) and Szöőr et al. (1966).

We have obtained considerably higher values for the specific activity of myosin cholinesterase of animals in the first 24 days of their postnatal life, and considerably lower values for the muscle of adult animals, than the values reported by Varga et al. (1957) which seem to be comparable to our data on the specific activity of myosin + actomyosin (Table 2).

The myosin fractions (Table 3, Figs 1a and 1b) were prepared with the method of Maercaud-Raeber et al. (1959) with some slight alterations and contained 2.5–3.0 S, 6.5–7.5 S, 11.0–12.0 S components in case of rabbits 1–24 days old. The actomyosin fraction contained 2.5–3.0 S, 6.5–7.5 S and 18–24 S components (Table 3, Fig. 1c). On the other hand, in the myosin and actomyosin fractions isolated from the muscle of adult animals one single fraction was detected with a sedimentation coefficient characteristic of myosin. Maercaud-Raeber et al. (1959) found 8–30 per cent of metamyosin (Raeber et al., 1955) by electrophoresis in the myosin fraction of the rabbit foetus prepared by the method of Mommaerts and Parrish (1951) which they claim cannot be detected or is only present in traces in the muscle of the adult animal.

Fig. 4 indicates the presence of bound nucleic acid or nucleotide, because for the myosin fraction the ratio of optical densities at 280 and 260 mμ is 0.87 and it is 0.884 for the actomyosin fraction. According to the Table compiled by Warburg and Christian (1941) this ratio of optical densities corresponds to 5 per cent nucleic acid. We believe that nucleic acid is in fact present in both the myosin and actomyosin fractions, since the optical density increases as a result of ribonuclease treatment (Fig. 5).

Pinaev (1965) studied the spectrophotometric and other properties of actomyosin fractions prepared from the muscle of rabbits of different ages. His absorption spectra (Pinaev, 1965, Fig. 3) show when evaluated as we have done above that the actomyosin of 10 days old rabbits contains 5 per cent nucleic acid, while that of the newly born and foetal rabbits contains 17–20 per cent nucleic acid. Maercaud-Raeber et al. (1959) published similar data on the isolated metamyosin of the rabbit foetus.

Ohshima et al. (1965) found somewhat less than 10 per cent nucleoprotein in the myosin B prepared from 11–14 days old chicken embryos.

There is an apparent contradiction between our present findings and the discussed data in the literature on the one hand, and the results of Bárány et al. (1965b) on the other hand. Bárány et al. prepared myosin from the muscle of two days old rabbits by the method of Portzehl et al. (1950), and then fractionated this preparation with ammonium sulphate according to Dubuisson (1946). This myosin appeared to be homogeneous when ultracentrifuged. The ratio of optical densities at 280 and 260 mμ was 1.68, indicating the absence of nucleic acid in the preparation.

We believe this difference to be due to the fact that both the aggregated myosin (11 – 12 S) and the 2.5 – 3.0 S component are removed by the fractionation with ammonium sulphate (Table 3), thus only the myosin component remains in solution, whose sedimentation coefficient is constant during the development of the animal (6.5 – 7.5 S in our studies, see Fig. 2; 6.5 – 7.2 S according to Ohshima et al. 1965 and Pinaev, 1965). Further support to this conclusion comes from the work of Trayer and Perry (1966), who also found no significant difference in both the sedimentation and the diffusion coefficients of the myosins prepared from the muscle of 28 days old and adult rabbits.

On the other hand, the characteristics of actomyosin are changed to a significant extent, for example, the size and the shape of the molecule certainly changes (Ohshima et al., 1965; Pinaev, 1965). Although the sedimentation coefficient of myosin remains unchanged, the presence of the 11 – 12 S component in the myosin fraction of the muscle of animals in the first 24 days of their postnatal life, and the absence of this component in the fully grown up animal indicates a certain change in the steric conformation and aggregational tendency of myosin in the course of ontogenesis. According to certain data in the literature the 11 – 12 S component would correspond to aggregated myosin (Portzehl et al., 1950; Szabolcs, Kövér, 1967).

The 2.5 – 3.0 S component which is present in both the myosin and actomyosin fractions during the first 24 days of postnatal life is missing from the muscle protein of the adult animal. The presence of this 2.5 – 3.0 S component can be demonstrated in the myosin, and even in the actomyosin fraction of the phylogenetically lower species, for example fish muscle (Kövér et al., 1963; Szabolcs et al., 1963). We have previously shown (Kövér et al., 1964) that a protein can be isolated from the myosin fraction of fish muscle with a sedimentation coefficient of $s^{\circ}_{20,w} = 2.83$ S, a molecular weight of 43 000 and a specific activity of 375 – 400 μM Ach per mg protein per hour. Since a component with a similar sedimentation coefficient was found in the rabbit muscle during the early stages of postnatal life, the possibility arises that this 2.5 – 3.0 S component might be partly responsible for the cholinesterase activity of our myosin and actomyosin fractions. It is, namely, a well-known fact (Beznák, 1944; Varga et al., 1954) that myosin does have some cholinesterase activity which can even be localized by electron microscopic histochemistry (Karnovsky, 1964).

No 2.5 – 3.0 S component was detected in the muscle of the adult animal, and so it would follow from our earlier discussion that the low cholinesterase activity of the myosin prepared from the muscle of the adult animal may be partly due to a change which the functional part of myosin undergoes during ontogenesis, but certainly also to the lack of the 2.5 – 3.0 S component as shown – and this point should be stressed – by ultracentrifugal experiments. That the functional part of myosin may undergo an alteration during ontogenesis is proved by the increasing ATPase activity in postnatal life (Varga et al., 1957; Bárány et al., 1965b; Pinaev, 1965; Ohshima et al., 1965) and such an alteration is certainly possible even though the sedimentation coefficient of myosin remains constant.

These results show that the contractile proteins of rabbit muscle undergo significant changes during ontogenesis development.

Similar results were obtained in a study of the biochemical properties of the sarcoplasmatic reticular fraction (Szabolcs et al., 1967).

Acknowledgement

We wish to thank Prof. Emil Varga for his generous support and valuable advices and Mrs. E. Csorba and Mr. I. Moldvai for their technical assistance.

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Shikimate:NADP Oxidoreductase in Barley Leaves

Purification and Properties

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Shikimate dehydrogenase (shikimate:NADP oxidoreductase (EC 1.1.1.25)) was extracted from first leaves of barley (Hordeum vulgare L.) seedlings and purified by ammonium sulphate fractionation and chromatography on a Sephadex G-200 column.

The pH optimum of enzyme activity was 9.9. The enzyme was specific for shikimate and NADP. p-Mercuribenzoate, borate and molybdate inhibited the enzyme activity. Ni^{2+}, Cu^{2+} and Mg^{2+} ions had no effect. Co^{2+} ions had a slight inhibitory effect. The enzyme was inhibited by high concentrations of cinnamic and quinic acids, by medium concentrations of coumarin and 4-OH-coumarin, and by low levels of o- and p-coumaric acids, ferulic acid, sinapic acid and aesculin. Low levels of cinnamic acid analogues (methoxy- and nitrocinnamic acids) also inhibited the enzyme. The aromatic amino acids had no effect on enzyme activity. The apparent K_M value of enzyme activity was found to be 0.37 mM for shikimic acid and 18.3 μM for NADP. The molecular weight of the enzyme, as determined by molecular sieving, was 40 000.

The shikimate dehydrogenase activity of crude extracts was resolved into three components by acrylamide disc electrophoresis. The purified preparation contained only one component.

Feeding of barley leaf tissues with various cinnamic acid derivatives had no effect on the level of shikimate dehydrogenase.

Introduction

The pathway of aromatic biosynthesis in higher plants has been clarified mainly by the use of isotope techniques (Neish, 1960; Pridham, 1965; Brown, 1966). Recently, enzymes participating in the pathway have also been isolated, purified and characterized from tissues of higher plants (Gamborg, 1966a, 1966b, 1967; Minamikawa et al., 1966; Sanderson, 1966). The rapid progress in this field makes a study of regulatory mechanisms possible. In the present paper a new method for the purification of shikimate dehydrogenase from barley leaves and some properties of the enzyme, including end-product inhibition, will be described.

Materials and Methods

Plant material

First leaves of 10 – 14-day old barley seedlings (Hungarian variety MFB) grown under ordinary greenhouse conditions were used as a starting material for enzyme isolation and purification.

Chemicals

Shikimic acid was obtained from Calbiochem (Los Angeles, Calif.), NAD, NADP and p-chloromercuribenzoate from Sigma Chemical Co. (St. Louis, Mo.), tyrosine, L-phenylalanine, dioxi-phenylalanine from Nutritional Biochemicals Corp. (Cleveland, Ohio) cinnamic acid and the cinnamic acid derivatives from Fluka Ag. (Buchs). The cinnamic acid analogues (m- and p-CH_3O-cinnamic acid, m- and p-NO_2-cinnamic acid) were kindly supplied by G. Matolcsy of the Research Institute for Plant Protection, Budapest. All the other chemicals were analytical grade reagents of Reanal Hungarian Fine Chemical Company.

Assays and analyses

The enzyme activity was assayed by measuring the increase in absorbance at 340 mμ in a Beckman DU-2 spectrophotometer. The reaction system contained 9.0 μmoles of shikimic acid, 1.0 μmole of NADP, 160 μmoles of glycine-NaOH buffer at pH 9.9 and a suitable amount of enzyme preparation in a final volume of 3.0 ml. In all experiments so much enzyme was added to the reaction mixture that the reaction rate remained linear with time for at least 5 minutes. Control cuvettes contained the complete assay system minus shikimic acid. When working with crude systems, in order to inhibit the reoxidation of $NADPH_2$, KCN was added to the reaction mixture in a final concentration of 10^{-3} M.

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumine as standard.

Electrophoretic techniques

The polyacrylamide disc electrophoresis technique of Ornstein (1964) and Davis (1964) was used to separate proteins of crude and/or purified fractions. Electrophoresis was performed at room temperature for 2.5 hours at 3 mA of constant current per gel tube. The detection of shikimate dehydrogenase after electrophoresis was carried out by incubating the gels in a reaction mixture containing the following ingredients per ml: shikimic acid 4 μmoles, $MgCl_2$ 4 μmoles, NADP 0.2 μmole, KCN 1.2 μmole, TRIS-HCl buffer, pH 7.5, 3.2 μmoles, p-nitroblue tetrazolium (NBT) 0.24 μmole, phenazine methosulphate 0.08 μmole. The gels were incubated with the reaction mixture until clearly observable bands developed. The reaction was then stopped with 7 per cent acetic acid. A control was always run in which the substrate or NADP was substituted by water to ascertain that the bands are due to a specific enzyme reaction.

Estimation of the molecular weight

The molecular weight of the purified enzyme was estimated by gel filtration on Sephadex G-100 and G-200 columns. Essentially the procedure of Andrews (1964) was followed. A detailed description of the calibration of the columns was given in a previous paper (Bagi, Farkas, 1967).

Results

Enzyme purification

30 grams of deep frozen leaf tissue were homogenized in 50 ml 0.2 M phosphate buffer, pH 7.5, in a mortar. The homogenate was passed through 4 layers of cheese cloth and centrifuged in a Janetzki Model K 60 refrigerated centrifuge at 20 000 g for 20 minutes. The supernate was subjected to fractionation by $(NH_4)_2SO_4$. Solid ammonium sulphate was added to the solution to 40 per cent saturation under constant stirring. The solution was kept in the cold for 30 minutes and centrifuged. The sediment was discarded. Solid $(NH_4)_2SO_4$ was added to the supernate to 45 per cent saturation. After full precipitation in the cold the solution was centrifuged and the sediment was dissolved in 4 ml

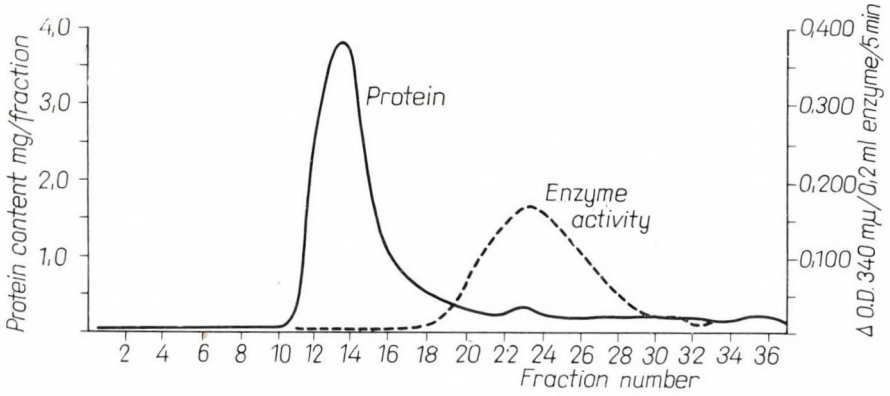

Fig. 1. Chromatography of shikimate dehydrogenase on a Sephadex G-200 column. The ammonium sulphate fraction obtained at 40—45 per cent saturation was used for the experiment

of 0.2 M phosphate buffer, pH 7.5. The undissolved protein was discarded. The protein solution was then transferred to a Sephadex G-200 column (25 cm × × 2.3 cm) previously equilibrated with 0.02 M phosphate buffer, pH 7.5. The proteins were eluted with the same buffer. 3 ml fractions were collected and assayed for enzyme activity (Fig. 1). The absorbance of the fractions was read at 280 mμ (protein content). Highly purified fractions were combined and stored at 0° C.

Properties of shikimate dehydrogenase

The enzyme activity was linear with time over a period of up to 10 minutes if the reaction rate was kept fairly low by applying sufficiently small enzyme amounts (O.D. 0.010 per minute in the assay system described). The purified enzyme gave no activity with quinic acid as substrate and showed an absolute requirement for NADP with shikimate as substrate.

As compared to the crude preparations the purified enzyme was relatively stable. About 5 per cent loss of activity was experienced per day during storage at 0°—4° C.

The dependence of enzyme activity on pH is shown in Fig. 2. The pH optimum was about 9.9.

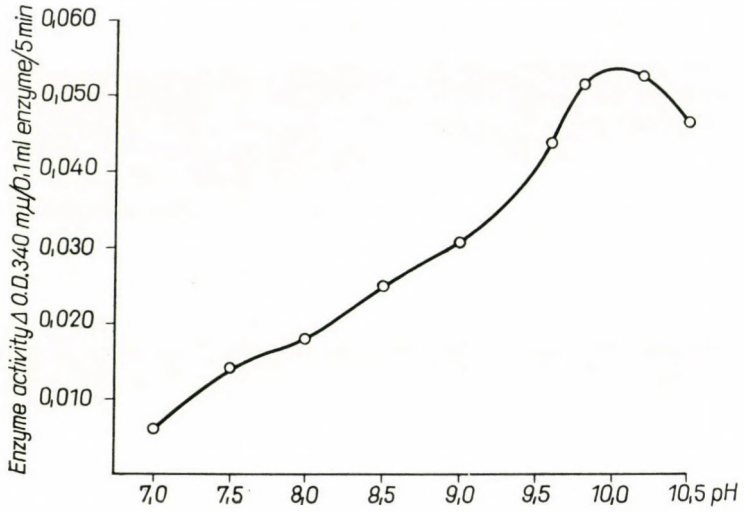

Fig. 2. Effect of pH on shikimate dehydrogenase activity. The assay conditions were the same as described in the methods except that 0.2 M Tris buffer was used in the pH range 7.0—9.0

As shown in Table 2 the enzyme was inhibited by p-chloromercuribenzoate. Cysteine and EDTA had no major influence on enzyme activity. Of the metal ions tested only Co^{2+} depressed the activity considerably. Molybdate and borate proved to be powerful inhibitors.

Table 1

Purification of shikimate:NADP oxidoreductase from barley leaf tissues

Fraction	Volume (ml)	Total activity (units*)	Total protein (mg)	Specific activity units/mg protein	Yield in per cent
Crude extract	120	119.9	460	0.26	100
$(NH_4)_2SO_4$-fraction 40—45 per cent saturation	4	35.5	27	1.30	29
Sephadex G-200 fraction No. 22—25	12	11.9	0.9	13.20	9.9

* Enzyme units are defined as the amount of enzyme which converts 1 μmole of substrate per minute.

The Michaelis constants for NADP and shikimate, calculated by the method of Lineweaver and Burk (cf. Dixon, Webb, 1964) were 18.3 μM and 0.37 mM, respectively.

Table 2

Effect of various compounds on the shikimate dehydrogenase of barley leaf tissues

The reaction mixture contained the standard ingredients as described in the methods + one of the compounds listed

Compound added	Concentration mM	Enzyme activity (control = 100 per cent)
p-chloromercuribenzoate	0.25	53
p-chloromercuribenzoate	0.5	19
cysteine	5.0	94
EDTA	5.0	89
$CoCl_2$	2.5	60
$NiCl_2$	2.5	95
$CuCl_2$	2.5	98
$MgSO_4$	2.5	98
$Na_2B_4O_7$	5.0	45
$Na_2B_4O_7$	25.0	14
Ammonium molybdate	2.5	20
Ammonium molybdate	5.0	10

The shikimate dehydrogenase activity of crude extracts was resolved into three distinct components by acrylamide disc electrophoresis (Fig. 3). In the purified preparation only one component was found which corresponded to the central band of the electropherogram of crude extracts (Fig. 4).

Table 3

Effect of various aromatic compounds on the purified shikimate dehydrogenase of barley leaf tissues

Compound added to the complete reaction mixture	Concentration (M)	Enzyme activity (control = 100 per cent)
Cinnamic acid	10^{-3}	100
Cinnamic acid	2.5×10^{-2}	59
Quinic acid	2.5×10^{-2}	62
p-Coumaric acid	5×10^{-4}	19
o-Coumaric acid	5×10^{-4}	89
Ferulic acid	5×10^{-4}	36
Sinapic acid	5×10^{-4}	37
Caffeic acid	10^{-4}	61
Tyrosine	10^{-3}	100
Phenylalanine	10^{-3}	100
Dioxyphenylalanine	10^{-3}	100
Aesculin	5×10^{-4}	18
Coumarin	10^{-2}	45
4-OH-coumarin	10^{-2}	59

The molecular weight of the enzyme, as determined by gel filtration, was approximately 40 000. The elution profile of marker proteins and purified barley shikimate dehydrogenase on a Sephadex G-100 column is shown in Fig. 5.

Inhibition studies

The effect of various aromatic compounds on the activity of the purified enzyme is shown in Table 3. A large variety of aromatic compounds, mostly cinnamic acid derivatives, inhibited the enzyme in relatively low concentrations. Tyrosine and phenylalanine were, however, ineffective. Methoxy- and nitro-

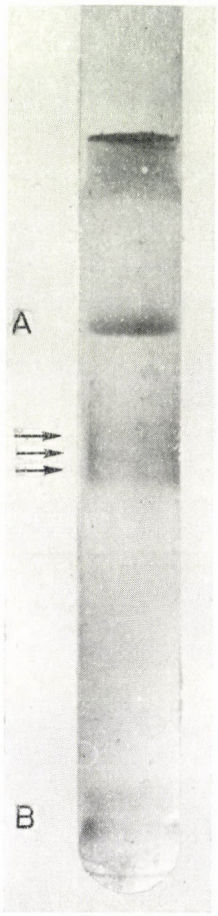

Fig. 3. Acrylamide gel electrophoretic pattern of the shikimate dehydrogenase activity of crude extracts. A = non-specific staining. B = indicator dye. The arrows indicate the position of the enzyme bands

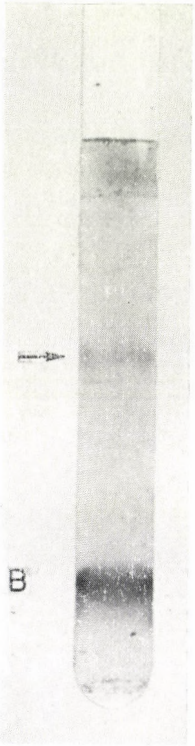

Fig. 4. Acrylamide gel electrophoretic pattern of shikimate dehydrogenase activity in the purified preparation. The arrow indicates the position of the enzyme band. B = indicator dye

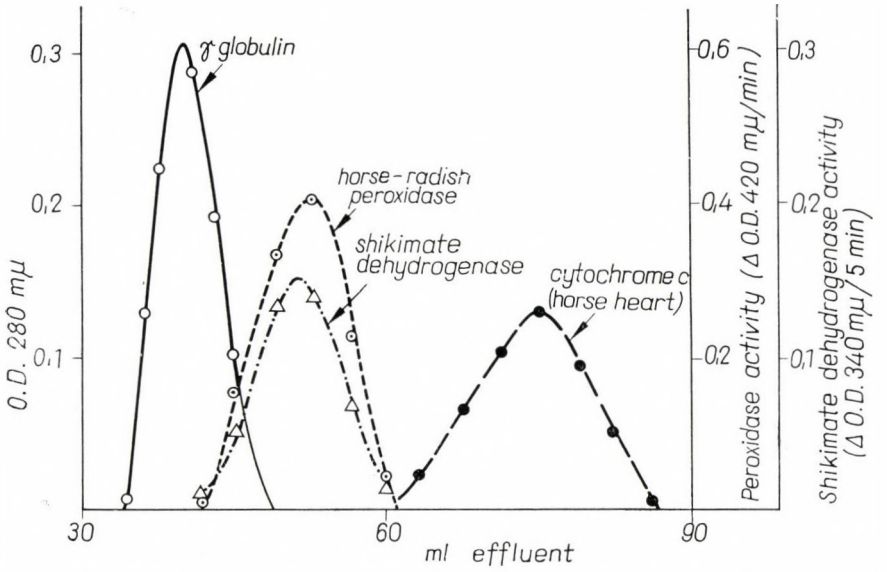

Fig. 5. Elution pattern of various marker proteins (globulin, Sigma Fraction II; horse-radish peroxidase, Worthington; cytochrome C, Sigma, Type II) and barley shikimate dehydrogenase on a Sephadex G-100 column. The distribution of globulin and cytochrome C was determined by measuring the absorbance at 280 mμ. The distribution of peroxidase and shikimate dehydrogenase was assayed by enzyme activity measurements. The system for peroxidase determination contained 0.5 ml of a 0.22 per cent guaiacol solution, 0.5 ml of 0.3 per cent H_2O_2, 0.2 ml effluent, 0.1 M Tris buffer at pH 7.5 in a total volume of 3.0 ml. Enzyme activity is expressed as increase in absorbance at 420 mμ. Shikimate dehydrogenase was assayed is described in the Methods. The molecular weight was estimated on the basis of the calibration curve published previously (Bagi, Farkas, 1967)

Table 4

The effect of cinnamic acid analogues on the activity of purified shikimate:NADP oxidoreductase

Enzyme activity was measured at substrate saturation

Inhibitor added to the system	Inhibitor concentration (M)	Enzyme activity expressed in per cent of the control
None	—	100
m-CH_3O-cinnamic acid	10^{-2}	71
p-CH_3O-cinnamic acid	10^{-2}	65
m-NO_2-cinnamic acid	4×10^{-3}	27
p-NO_2-cinnamic acid	4×10^{-3}	46

derivatives of cinnamic acid (synthetic cinnamic acid analogues) also inhibited the enzyme activity (Table 4).

The type of inhibition was investigated in kinetic studies by the Lineweaver-Burk method (cf. Dixon, Webb, 1964). Competitive type of inhibition was proved for cinnamic acid, 4-OH-coumarin and aesculin. In Fig. 6 examples of the kinetic analysis are given. Of the synthetic cinnamic acid analogues investigated, the methoxy compounds inhibited the enzyme competitively (Fig. 7) the nitro-derivatives, however, proved to be non-competitive inhibitors (Fig. 8).

For reasons to be discussed later the type of inhibition could be less unequivocally determined with the following compounds: o- and p-coumaric acids, ferulic acid, sinapic acid, chlorogenic acid, caffeic acid.

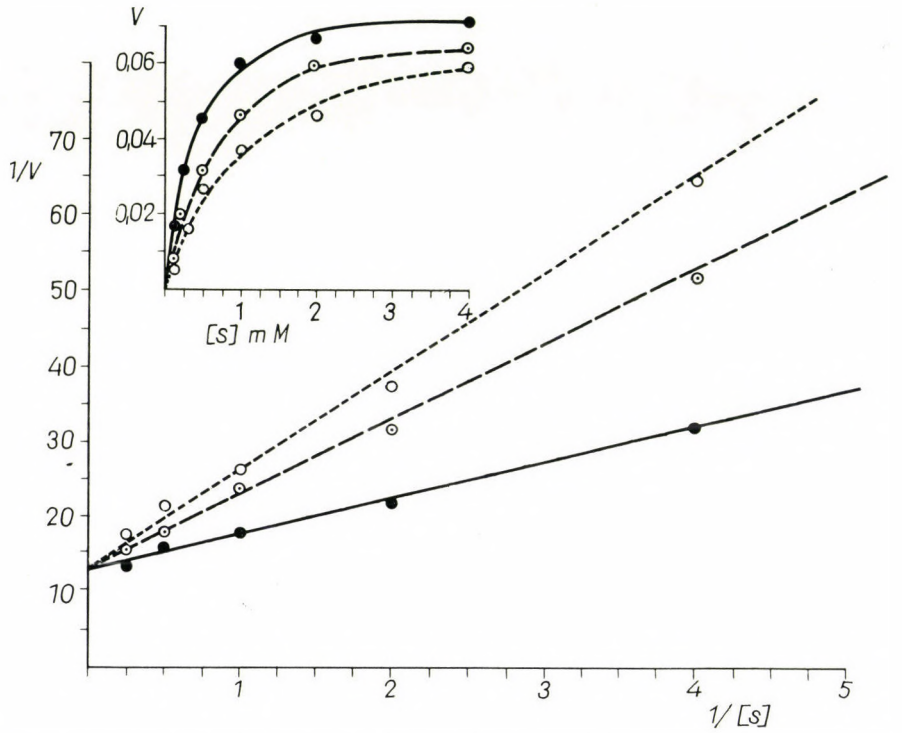

Fig. 6. Competitive inhibition of shikimate dehydrogenase by cinnamic acid and 4-OH-coumarin. Lineweaver-Burk plot of the rate of reduction of NADP by the enzyme in the presence of increasing concentrations of shikimic acid in the absence of inhibitor ○ — ○ — ○ and in the presence of 5×10^{-3} M 4-OH-coumarin ⊙ — ⊙ — ⊙ or 1.5×10^{-2} M cinnamic acid ● — ● — ●. The assay system contained the ingredients described in Methods

Discussion

Several enzymes of the shikimic acid pathway have recently been isolated in an effort to establish the enzymic reactions involved in aromatic biosynthesis in higher plants. Shikimate dehydrogenase, the third enzyme of the pathway, has been found to occur in several plant species (Nandy, Ganguly, 1961; Balinsky,

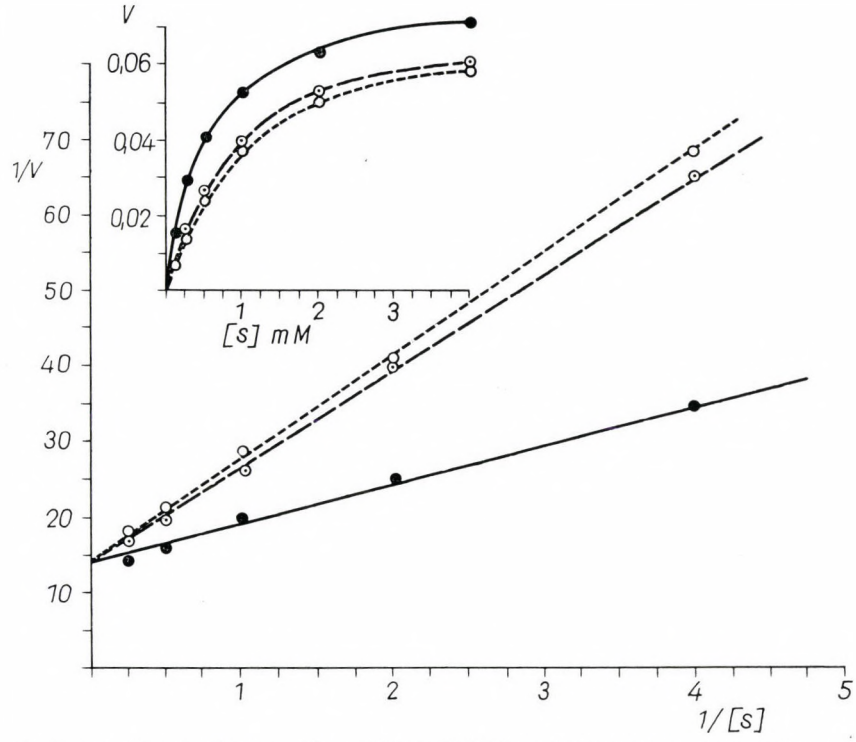

Fig. 7. Competitive inhibition of shikimate:NADP oxidoreductase by p-methoxy- and m-methoxy-cinnamic acids. Lineweaver-Burk plot of enzyme activity. No inhibitor in the reaction mixture ○ — ○ — ○. 5×10^{-3} M p-CH_3O-cinnamic acid ⊙ — ⊙ — ⊙ or 5×10^{-3} M m-CH_3O-cinnamic acid ● — ● — ● in the reaction mixture.The assay system contained the ingredients described in the Methods + one of the inhibitors

Davies, 1961; Gamborg, 1966a, 1967; Minamikawa et al., 1966; Sanderson, 1966; Higuchi, Shimada, 1967). However, detailed report on a reasonably purified preparation is only available on the enzyme from etiolated pea epicotyls (Balinsky, Davies, 1961). In the present paper a new 2-step purification procedure is described which consists of $(NH_4)_2SO_4$-fractionation of crude extracts from barley leaves followed by gel filtration. The purification procedure is highly reproducible due to the extraordinarily good separation of the peak of shikimate oxidase activity from the bulk of protein (Fig. 1).

The easiness of the extraction of shikimate dehydrogenase from barley leaves by the procedure described is to be explained by the apparently low amounts of inhibitory substances (presumably phenolics) present in the barley leaf tissues. This is indicated by the observation that the yield of enzyme activity in the crude preparation was the same both in the presence and in the absence of polyvinylpyrrolidone, a compound known to bind phenolics during extraction. Sanderson (1966) succeeded in demonstrating shikimate dehydrogenase activity in the tea shoot only if the extraction was done in the presence of polyvinyl-

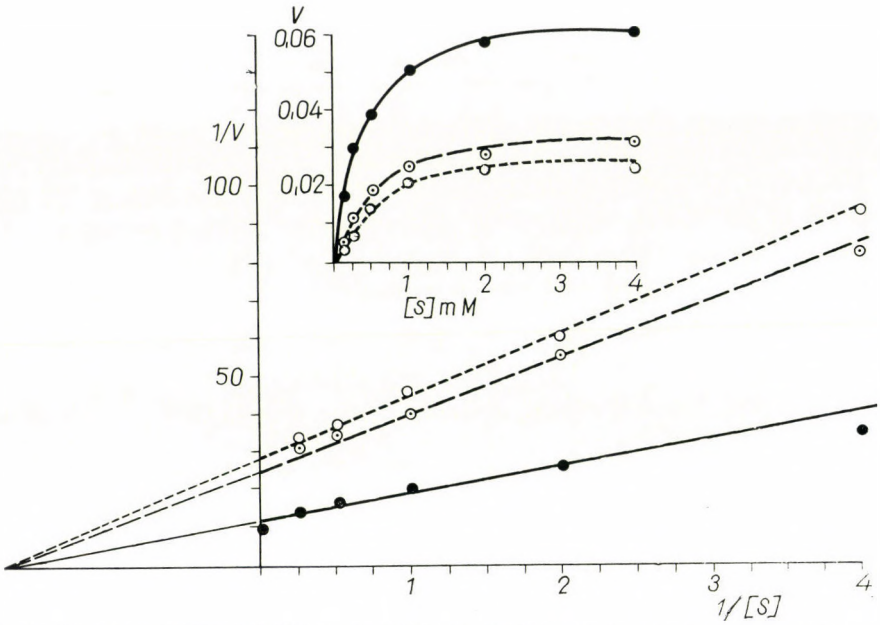

Fig. 8. Non-competitive inhibition of shikimate:NADP oxidoreductase by m-NO_2- and p-NO_2-cinnamic acids. Lineweaver-Burk plot of enzyme activity. No inhibitori n the reaction mixture ○ — ○ — ○ 4×10^{-4} M m-NO_2-cinnamic acid ⊙ — ⊙ — ⊙ or 4×10^{-4} M p-NO_2-cinnamic acid ● — ● — ● in the reaction mixture. The assay system contained the ingredients described in the Methods + one of the inhibitors

pyrrolidone. Similarly, we detected shikimate dehydrogenase activity in tobacco leaf extracts only if the tissues were extracted in the presence of high amounts of polyvinylpyrrolidone (0.6 g polyvinylpyrrolidone/1 g fr. wt.). Most probably, much enzyme activity is lost during extraction in these cases, even in the presence of polyvinylpyrrolidone.

As revealed by acrylamide disc electrophoresis the shikimate dehydrogenase is present in the crude extract of barley leaves in multiple forms. It is not known at present whether the electrophoretic variants have different enzymological properties, since the purified preparation only contained one of the components.

As judged from the effect of p-chloromercuribenzoate, the enzyme appears to have essential thiol groups and no metal-ion requirement. The inhibition by borate and molybdate can be explained by an effect on the substrate rather than on the enzyme (borate and molybdate are known to form complexes with hydroxy acids). These properties of the enzyme and its high pH optimum are in line with earlier observations and seem to indicate that the shikimate dehydrogenases isolated from different sources are very similar (cf. Balinsky, Davies, 1961; Sanderson, 1966). It is certainly interesting that the above characteristics of shikimate dehydrogenase are common with those of quinate dehydrogenase (Gamborg, 1966a), another enzyme the participation of which is implicated as a side reaction of the shikimate pathway.

Perhaps the most remarkable property of shikimate dehydrogenase is its sensitivity towards a number of compounds which are known to be intermediates or end-products of aromatic biosynthesis. It has been shown by Balinsky and Davies (1961) that the shikimate dehydrogenase of pea epicotyls is competitively inhibited by substituted benzoic acids. The quinate dehydrogenase of mung bean cell suspension cultures exhibited the same property (Gamborg, 1966a). The present work has shown that cinnamic acid and a large variety of cinnamic acid derivatives are also potent inhibitors of shikimate dehydrogenase. Since naturally occurring end-products of the shikimic acid pathway are involved, the idea seems to be logical that these compounds might exert a feedback-type regulatory effect on the pathway. The concentration of many cinnamic acid derivatives in the plant tissues is high enough to be compatible with this hypothesis.

The type of inhibition was studied by kinetic analysis. Competitive inhibition was established for cinnamic acid, 4-OH-coumarin and aesculin. Difficulties were encountered in carrying out the kinetic analysis with the hydroxycinnamic acids, because illumination with UV light results in a trans → cis-conversion. The new equilibrium is reached slowly and its position is dependent, among others, on the concentration of the compound under investigation (Haskins et al., 1964; Kahnt, 1966, 1967). Since the cis- and trans-hydroxycinnamic acids have different UV spectra the trans → cis-conversion, which takes place in the cuvette during measurement, greatly interferes with the assay.

It is interesting to note that not only cinnamic acid but also the methoxylated cinnamic acid derivatives inhibit the enzyme. These compounds do not occur in nature but they are closely related to naturally occurring methoxylated hydroxycinnamic acid derivatives. As in contrast to cinnamic acid the methoxy derivatives are known to be metabolically inert to a high extent (Farkas, Matolcsy, 1961; Bland, Logan, 1967) they might find an application as metabolic inhibitors of the shikimic acid pathway. The nitro derivatives proved to be even more powerful inhibitors of the enzyme than the methoxy-cinnamic acids. However, they are more toxic if supplied to plant tissues *in vivo*.

The regulation of aromatic biosynthesis is better understood with microorganisms than with higher plants. Allosteric effects, repression and induction have been described as regulatory mechanisms involved in aromatic biosynthesis in microorganisms (Doy, Brown, 1965; Cotton, Gibson, 1965; Pittard, Wallace, 1966; Brown, Maas, 1966; Lingens et al., 1966, 1967a, b). The cinnamic acid derivatives apparently do not act as repressors of shikimate dehydrogenase in the barley leaf tissues. In feeding experiments the various "end-products" of the shikimic acid pathway did not alter the level of shikimate dehydrogenase. Thus, the inhibitory effects discussed above are the first indications of a possible regulatory mechanism in higher plant tissues.

Acknowledgement

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Endoplasmic Units

Non-Reacting Globular Intracisternal Particles of the Endoplasmic Reticulum, Revealed by an Electron Histochemical Enzyme Reaction as a Negative Stain

(Short Communication)

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Acetylcholinesterase reaction of cholinergic nerve cells reveals the regular occurrence of 320—350 Å non-reacting globular particles (endoplasmic units) within the cisternae of the endoplasmic reticulum. Based on the electron microscopic localization of acetylcholinesterase activity in the neuronal perikaryon, the hypothetical explanation is forwarded that endoplasmic units may take part in the final modelling of the tertiary structure of protein molecules.

Introduction

The endoplasmic reticulum, identified as an electron microscopic equivalent of the ancient histologists' "basophilic ergastoplasm" and the biochemists' "microsomal fraction" (Porter, 1953; Palade, Porter, 1954; Palade, Siekevitz, 1956) represents the cellular machinery of protein synthesis. Ribosomes, located near to the lateral surfaces of the endoplasmic cisternae, are considered to be those strategic points ("typewriters") on the surfaces on which the sequential arrangement of amino acids takes place in the preferential order prescribed by the triplet-coded information carried by messenger-RNA molecules. Accordingly, intracisternal lumina of the endoplasmic reticulum would only appear to be of secondary importance, taking part in the collection of freshly synthesized protein molecules.

Fine structural localization of acetylcholinesterase activity in the cytoplasm of cholinergic nerve cells, however, suggests an active participation of endoplasmic cisternae in the process of protein synthesis. Some indications of this suggestion obtained in ultrastructural studies will be briefly described in this paper.

Materials and Methods

Fine structural localization of acetylcholinesterase (AChE) has been studied in the feline cervical superius ganglion by means of an electron histochemical technique. Ganglia were fixed by perfusion through the carotid artery under

hexobarbital anesthesia, using a cacodylate-buffered 10 per cent formaldehyde solution, containing 0.2 M sucrose. After 50 minutes perfusion, the ganglia were removed and post-fixed in the same fixative for 2 hours at 4° C. The samples were rinsed in cacodylate-sucrose for 4 hours and 50 μ sections were cut on a freezing microtome. Incubation of these sections has been carried out at 4°C for 1–4 hours in the following solution, slightly modified after Koelle (1954) and based upon our earlier studies (Csillik et al., 1966; Kása, Csillik, 1966):

acetylthiocholine iodide	7 mg
sodium acetate, 0.1 M	2.2 ml
acetic acid, 0.1 M	0.1 ml
copper glycinate, 0.2 M	0.1 ml
uranyl acetate, 0.1 M	0.1 ml

Control sections for enzyme inhibition were preincubated in Mipafox (10^{-6} M) or in BW 284 C 51 (10^{-4} M).

After incubation, the samples were rinsed in 0.25 M sucrose (5 minutes) and transferred into an isotonic yellow ammonium sulphide solution (made isohydric by adding 0.1 M acetic acid and then centrifuged). This latter treatment is necessary to induce conversion of the metallic end product of the enzyme reaction (copper-uranyl-thiocholine) into the corresponding mercaptide. Finally, the samples were post-fixed in osmic acid, rinsed, dehydrated in graded alcohols and embedded in Durcupan (Fluka).

Thin sections were obtained on LKB and Tesla Ultrotomes, using glass knives. The sections were partly stained with lead citrate (Reynolds, 1963) and inspected under Tesla 242 D and 413 electron microscopes.

Results

In those few nerve cells that display an AChE activity in the feline ggl. cervicale superius (Fig. 1), the enzyme reaction outlines the Nissl bodies. Under the electron microscope (Fig. 2) AChE activity appears to be confined to the endoplasmic reticulum, the electron dense reaction product filling the cisternae. No activity could be seen extracisternally: ribosomal and mitochondrial elements are free of the reaction product (Fig. 3).

In those cisternae that happen to appear in a tangential plane of the section, the reaction product is inhomogeneous. In such cases, the electron dense end product outlines "white", i.e. non-reacting globular units, measuring 320–350 Å in diameter (Fig. 4). Once identified, the regular occurrence of such non-reacting units can readily be observed in transverse sections of cisternae, too, (Fig. 5) especially if the enzyme reaction (the "negative staining") is less strong.

Discussion

The very nature of the globular particles within the endoplasmic cisternae, that can conveniently be called "endoplasmic units", is unknown at present (Fig. 6). The fact that they have not been disclosed by means of regular electron

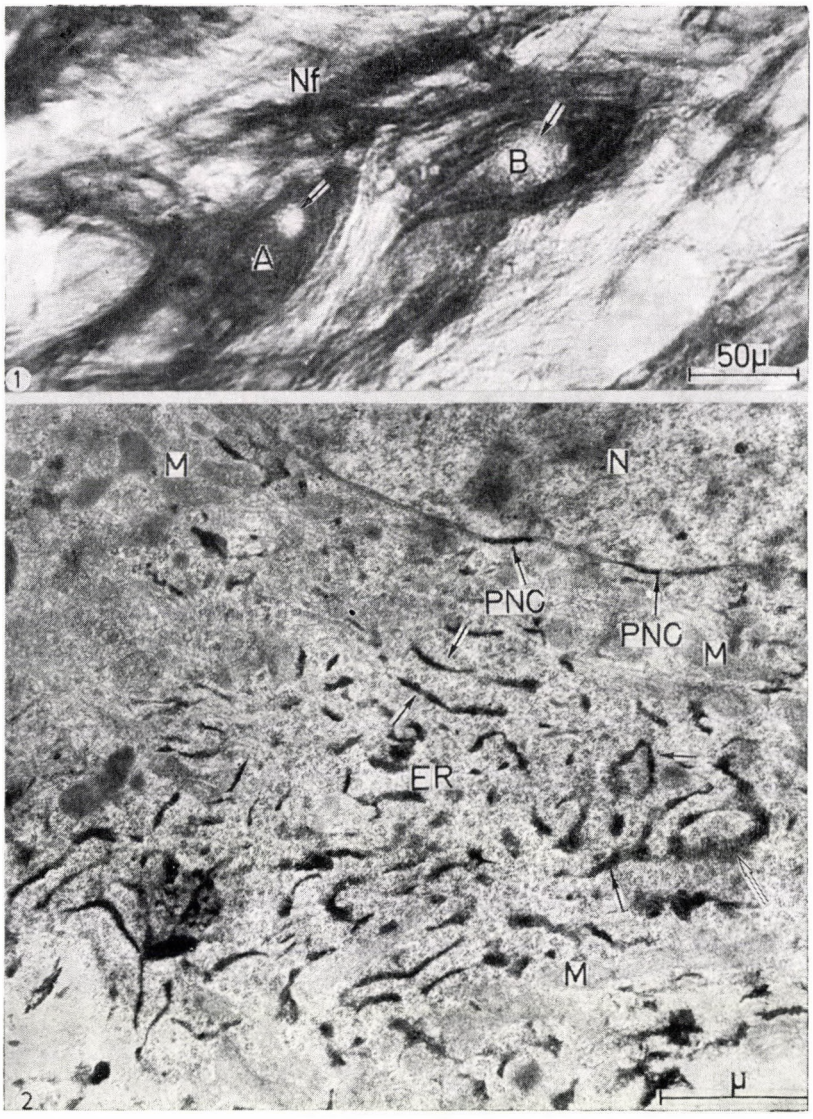

Fig. 1. AChE reaction in the feline ggl. cervicale superius. Cryostat section, acetylthiocholine medium according to Koelle. A: strongly reacting ganglion cell; B: weakly reacting ganglion cell. Arrows point at nuclei. Nf: nerve fibers, exerting a strong AChE reaction in the intercellular space. Arrows point at cell nuclei

Fig. 2. AChE reaction in a ganglion cell. N: nucleus. PNC: AChE reaction in the perinuclear cisterna; M: mitochondria (non-reacting); ER: endoplasmic reticulum (Nissl substance); arrows point at endoplasmic cisternae filled by the enzyme reaction product. Double arrow points at the tangential section of a cistern, shown under higher power in Fig. 4

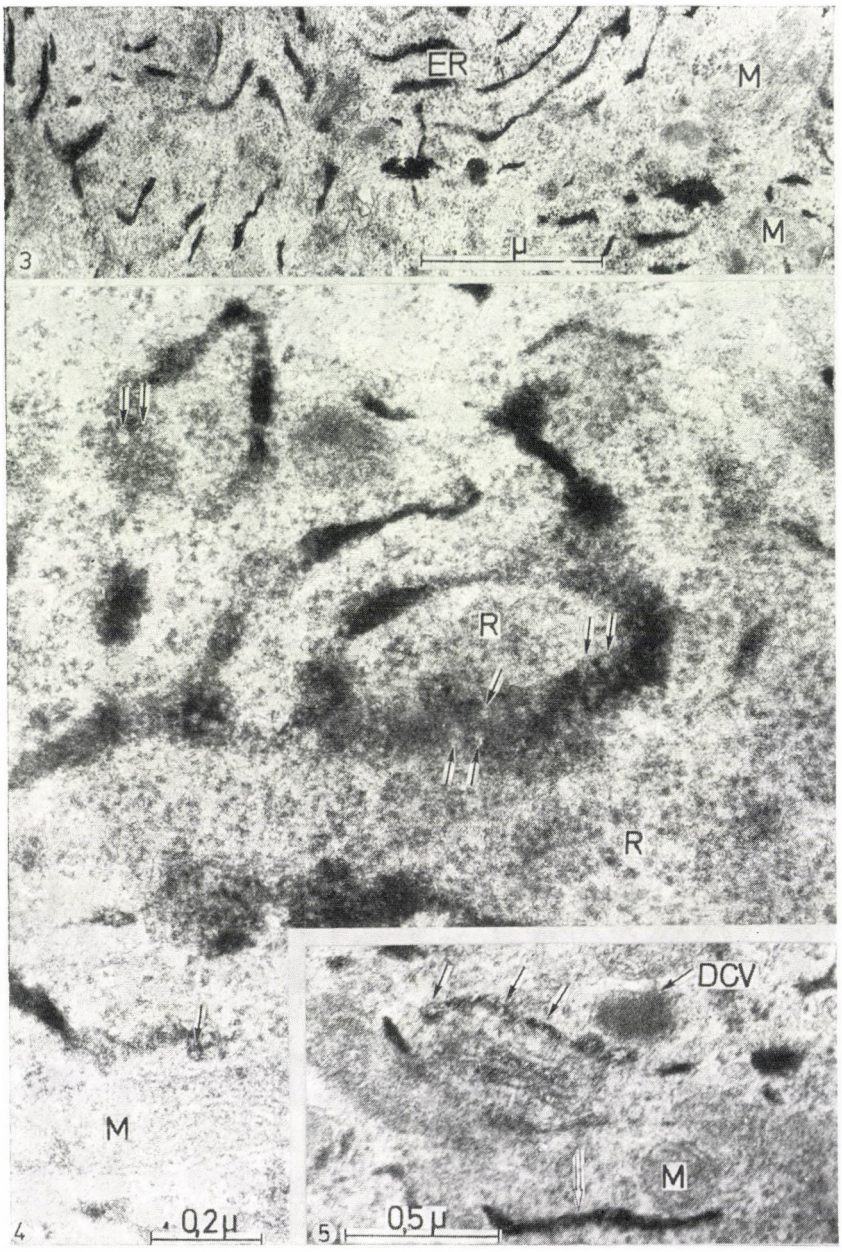

Fig. 3. Nissl substance in a ganglion cell. Note the heavy electron dense deposit (AChE activity) in the cisternae of the endoplasmic reticulum (ER) and the non-reacting ribosomes around the cisternae. M: mitochondrion

Fig. 4. High power view of the AChE reaction in the endoplasmic reticulum in tangentially cut cisternae. Note the frequent occurrence of 320—350 Å non-reacting endoplasmic units (arrows) outlined by the electron dense reaction product ("negative stain") of the AChE reaction. R: ribosomes; M: mitochondrion

microscopic techniques suggests that their chemical composition is different from that of "regular" (unit membrane-bound) cytological structures. In other words, their low osmiophilia appears to be due to the lack of those chemical moieties that are responsible for the usual osmic acid staining of other intracellular particles.

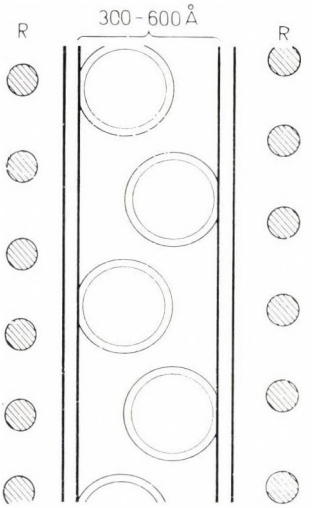

Fig. 6. Highly schematic pattern of the endoplasmic reticulum. Surrounded by 150 Å ribosomes (R), the cistern of the endoplasmic reticulum measuring 300—600 Å in width, is depicted. The lumen of the cistern contains 320—350 Å endoplasmic units

With regards to their *staining properties*, endoplasmic units show a striking similarity to the electron transport particles on mitochondrial cristae (Fernández-Morán, 1964). Electron transport particles become visible only after using a negative staining procedure. In an analogous manner, endoplasmic units become visible only after using the AChE staining reaction; in this respect the end product of the enzyme reaction actually plays the role of an electron dense "negative stain".

Protein synthesis, i.e. the sequential coupling of individual amino acids, takes place on the surface of ribosomes. The primary structure (the sequential order) also determines the secondary conformation. Yet the fact that freshly synthetized AChE molecules exhibit an enzyme reaction only within the endoplasmic cisternae, suggests that a final modelling, probably the elaboration of the tertiary configuration which is responsible for the enzyme action, of the protein molecule is only accomplished within the lumina of the endoplasmic cisternae. We, therefore, suggest that endoplasmic units may play a part in this „final touch" of protein synthesis.

Fig. 5. Cross section of endoplasmic cisternae, containing AChE reaction product. Arrows point at non-reacting endoplasmic units in a less strongly reacting cistern. Double arrow points at the heavy reaction in another cistern, where the profiles of the endoplasmic units are less obvious. DCV: dense-core vesicle; M: mitochondrion

The occurrence of endoplasmic units is not restricted to cholinergic nerve cells but it is rather a common and general feature of the endoplasmic reticulum. In well-stained ultrathin sections of entirely different cell types (e.g. in exocrine pancreas cells, as seen in Don Fawcett's recent atlas, 1966) the faint outlines of endoplasmic units can be observed. The chemical characterization of endoplasmic units, also observed occasionally after differential centrifugation (Adams, D. H., personal communication) remains to be solved by biochemical techniques.

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Amino Acid Sequence around the Reactive SH-Group of α-Glycerophosphate Dehydrogenase

(Short Communication)

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α-Glycerophosphate dehydrogenase (GDH) catalyzes the reaction between triosephosphates and NADH (Telegdi, 1968). There are contradictory data about the participation of the SH-groups of GDH in this catalytic function. Van Eys et al. (1959) have shown a complete inactivation by blocking 1 SH-group in GDH, and suggested that there is one active center in the molecule. On the other hand no essential SH-group was found in GDH by Nagradova (1959).

We have already dealt with the role of SH-groups in rabbit muscle GDH in previous papers (Telegdi, Keleti, 1964, 1966). There is convincing evidence in these papers for the participation of SH-groups in the reaction catalyzed by GDH.

The aim of the present work was to identify the essential SH-group and to determine the amino acid sequence of the peptide which contains this group.

Highly purified crystalline GDH was isolated from rabbit muscle by a method described earlier (Telegdi, 1964).

The effect of bromoacetic acid

Carboxymethylation was carried out by adding 20 to 600 mole equivalents of ^{14}C-bromoacetate to the enzyme in 0.1 M phosphate buffer, pH 7.0 at room temperature. The amount of bromoacetate bound to enzyme was checked by titrating with p-hydroxymercuribenzoate (p-HMB) the residual free SH-groups after gel-filtration and by measuring the radioactivity of the carboxymethylated enzyme. The number of total SH-groups of the GDH preparations was determined before each experiment and it was used as reference.

The reaction with bromoacetate takes place in 4–18 hours depending on the concentration of the reactants and on the pH of the solution. At the end of the reaction 1.4 to 2.0 equivalents of bromoacetate could be determined bound to the enzyme (Table 1).

The number of bromoacetate molecules reacted, as measured by the radioactivity of the protein, was equivalent to the decrease in the number of SH-groups. It follows, that bromoacetate only reacted with some SH-groups in the native enzyme, and no reaction with other amino acid residues occurred. The carboxymethylation of these SH-groups resulted in the loss of 70–75 per cent

of the enzymic activity. From these data it seems possible that at least two SH-groups are involved in the catalytic activity of GDH.

Table 1

Effect of bromoacetate on GDH
Each sample was incubated for 8 hours in 0.1 M phosphate buffer, pH 7.0
GDH: 3.2×10^{-3} μmole/ml

Number of free SH-groups titrated with p-HMB		Mole equ. of bromo-acetate bound	Enzymic activity in per cent of original	
Native	Enzyme + 20 equ. bromo-acetate		Native GDH	Enzyme + 20 equ. bromo-acetate
11.6	10.2	1.4	96	30
12.4	10.8	1.6	92	26
12.2	10.2	2.0	92	23

Isolation of peptides containing ^{14}C-carboxymethyl cysteine

In order to isolate the radioactive peptides, 81 mg of GDH were dissolved in 0.1 M phosphate buffer, pH 7.0 and 32 mg of ^{14}C-bromoacetate (200 mole equivalents) were added. This solution was incubated at room temperature for 18 hours. Under these conditions 2.0 mole equivalents of ^{14}C-bromoacetate were bound to the enzyme. The excess of ^{14}C-bromoacetate was removed by the addition of 0.1 ml of 15 M mercaptoethanol, followed by gel-filtration on a Sephadex G-50 column. The enzyme solution was then reacted with an excess of non-radioactive bromoacetate in the presence of 8 M urea. The solution was incubated for 16 hours at room temperature. After incubation the enzyme solution was dialyzed against tap water overnight. The precipitate formed during dialysis was washed several times with distilled water and acetone, and then it was suspended in water to 1 per cent final concentration and digested with 1/30 weight of trypsin at pH 8.0 in a Radiometer TTT1 type autotitrator. After the rate of digestion fell off, a further 1/30 part of trypsin was added to complete the digestion. Trypsin was then heat-denatured and the hydrolysate filtered and freeze-dried. The material thus obtained was dissolved in 0.05 N NH_4OH and gel-filtered on a 1×120 cm Sephadex G-25 column. The column was eluted with 0.005 N NH_4OH. The fractions containing radioactivity emerged in a single peak. This radioactive component was recovered by freeze-drying, dissolved in water and submitted to a micro-preparative paper-electrophoresis. The electrophoresis was carried out in 1.5 hours at pH 5.0 (acetic acid : pyridine : water, 1 : 1 : 100) with 1100 V. The radioactivity was revealed by autoradiography. A slightly acidic fraction moving toward the anode at pH 5.0 contained most of the radioactivity. This fraction was eluted with 0.1 N NH_4OH and, for further

purification, after freeze-drying, it was submitted to electrophoresis at pH 1.9 (formic acid : acetic acid : water, 5 : 15 : 100; with 1100 V, 1.5 hours). Electrophoresis was followed by autoradiography. The radioactive band was eluted with 0.1 N NH_4OH and its homogeneity was checked by with chromatography (pyridine : amyl-alcohol : water, 35 : 35 : 30).

Amino acid composition and sequence of the radioactive peptide

The eluted radioactive peptide was hydrolyzed with 6 N HCl, at 105° C in 16 hours. The amino acid composition of the hydrolysate was determined in an amino acid analyser of Bender-Hobein type. The following composition was obtained:

^{14}CM-Cys$_1$, Lys$_1$, Phe$_1$, Asx$_1$, Thr$_1$, Ser$_1$, Glx$_1$, Gly$_2$, Ala$_1$, Val$_{1-2}$, Ile$_1$, Leu$_1$

The N-terminal amino acid was found to be Phe.*

In order to determine the amino acid sequence, the peptide was digested with subtilisin, at pH 8.0 and 37° C, in 1.5 hours. The hydrolysate was submitted to electrophoresis at pH 6.5 – 5.0 (acetic acid-pyridine-water) and chromatography (pyridine-amyl alcohol-water). 0.005 per cent ninhydrin was used to develop the peptides. After elution and freeze-drying, an aliquot of the peptides was hydrolyzed with 6 N HCl, at 105° C, for 16 hours, and their amino acid composition was determined. Another sample was used for the determination of the terminal amino acids by the dansyl method of Gray and Hartley (1963). The dansyl-amino acids were identified by electrophoresis.

The following di- and tripeptides were obtained after the isolation of the peptides obtained by digestion with subtilisin:

Phe. (Ala, Val)
Ala. Val
Asx. (Thr, ^{14}CM – Cys)
Thr. ^{14}CM – Cys, Ser)
Ser. Gly
Gly. (Glx, Gly)
Gly. Glx
Ile. Val$_{0-1}$ (Leu, Lys)

Phe. Ala. Val. (Val$_{0-1}$). Asx. Thr. ^{14}CM – Cys. Ser. Gly. (Glx, Gly). Ile. (Val$_{0-1}$. Leu). Lys

Since a single peptide containing ^{14}CM-Cys was obtained it shows that ^{14}C-bromoacetate was bound to the SH-group of two identical peptides. This would agree with the existence of two subunits in GDH, as found in sedimentation (Deal, Holleman, 1964) and coenzyme binding studies (Pfleiderer, Auricchio, 1964; Telegdi, Keleti, 1968).

In the presence of 8 M urea 5 further SH-peptides can be shown in the tryptic hydrolysate. Accordingly, there are six different SH-peptides in each of the two identical or similar subunits of GDH.

* Since this work was done, we became aware that it would be necessary to exclude Ile as the N-terminal amino acid. This work is in progress.

There is some similarity between the peptides containing the reactive SH-group of D-glyceraldehyde-3-phosphate dehydrogenase (GAPD) and GDH:

Asn. Ala. Ser. CMC. Thr. Thr. Asn. Cys.	(GAPD: Harris et al., 1963)
Val. Asx. Thr. CMC. Ser. Gly. (Glx, Gly)	(GDH: this work)

The reactive SH-group of GAPD is known to be involved in the binding of the intermediate of D-glyceraldehyde-3-phosphate (GAP) oxidation (Harris et al., 1963). GDH also catalyzed the transformation of GAP (as well as of dihydroxyacetonephosphate) through the formation of an intermediate product (Telegdi, 1968). It may be assumed that the reactive SH-group of GDH – similarly to that of GAPD – is involved in the binding of this intermediate, since it is known that the essential SH-groups of GDH do not bind the substrates or the coenzymes (Telegdi, Keleti, 1966).

Acknowledgements

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Isolation of Porcine β-Lipotropic Hormone

(Preliminary Report)

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(Received January 17, 1968)

Li and coworkers (1965, 1966) isolated a polypeptide hormone (β-LPH*) from sheep adenohypophysis and they also established the total amino acid sequence of this polypeptide. Recently, the same preparative procedure was successfully applied to the isolation of the hormone from beef hypophysis; there was no apparent difference in the amino acid composition and biological characteristics of the two hormones (Lohmar, Li, 1967).

Preparations with specific lipotropic effect have previously been isolated from hog hypophysis (Rudman et al., 1960; Astwood et al., 1961), however, the active peptides of these preparations appear to be basically different from β-LPH (Rudman et al., 1961; Friesen, 1964; Hofman, Serdarevich, 1963).

The question arises whether an analogue of the above-mentioned β-LPH exists in hog hypophysis. If so, it seems to be worth-while from a molecular genetical point of view, to compare this peptide to β-LPHs isolated from sheep and beef.

Our purification procedure was the same as that used by Li and coworkers (1966) for the purification of sheep β-LPH up to the preparation of fraction D′. 5000 pieces of porcine adenohypophysis gave 5.2 g of fraction D′. Fig. 1 shows the separation of 700 mg of fraction D′ on CMC column. β-LPH was assigned to the peak which showed *in vitro* lipolytic activity on rabbit retroperitoneal fat tissue and no appreciable corticotropic activity as measured by Sayers s.c. test. The active peptides of the last two peaks were identified as corticotropin A_2 and A_1, resp., by polyacrylamide gel electrophoresis and amino acid analysis.

50 mg lyophilized substance of the β-LPH peak was rechromatographed on a second CMC column under similar conditions. By this last purification step 30 mg β-LPH was obtained. The homogeneity of the peptide was proved by ultracentrifugal analysis and polyacrylamide gel electrophoresis. Ultracentrifugation was performed with a MOM G 120 apparatus. The weight average molecular weight of the preparation was 11 800 ± 500, as calculated from equilibrium mass distribution in a 0.5 per cent solution of the polypeptide in 0.05 N HCl by taking 0.730 for partial specific volume which, in turn, was calculated from the amino acid composition (Cohn, 1943). Fig. 2 shows the purity and electrophoretic mobility of the final β-LPH preparation compared to fraction D′.

1.5—2 mg samples of peptide hydrolysates were analysed with the aid of an amino acid analyzer, type EEL (6.7 N HCl, 24 hours, 105° C in evacuated

* β-LPH = β-lipotropic hormone.

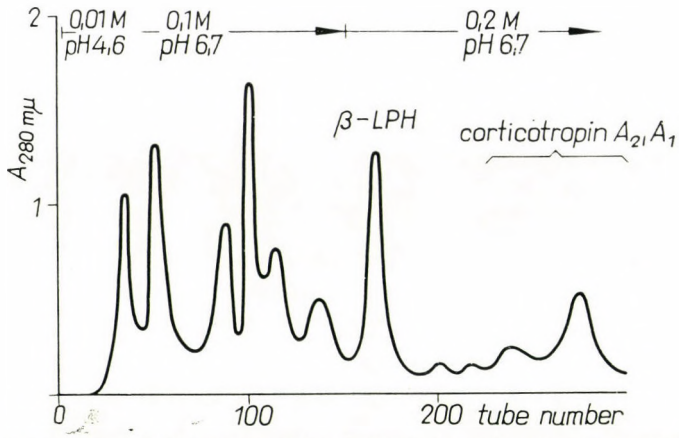

Fig. 1. Chromatography of the fraction D′ on CM-cellulose. The size of the CMC (Whatman CM 11) column was 2.2×60 cm, it was equilibrated with 0.01 M ammonium acetate buffer (pH 4.6). Gradient elution was started as soon as the column had been loaded with 700 mg of fraction D′, by mixing 0.1 M pH 6.7 ammonium acetate buffer to the starting buffer (0.01 M, pH 4.6) in a mixing vessel of a volume of 500 ml. The buffer in the upper vessel was replaced by 0.2 M pH 6.7 ammonium acetate buffer at fraction 150. The flow rate was 60 ml/hr. The abscissa shows the number of 5-ml fractions, the ordinate gives light absorption at 280 mμ with 1 cm optical cells. The pooled peaks were lyophilized

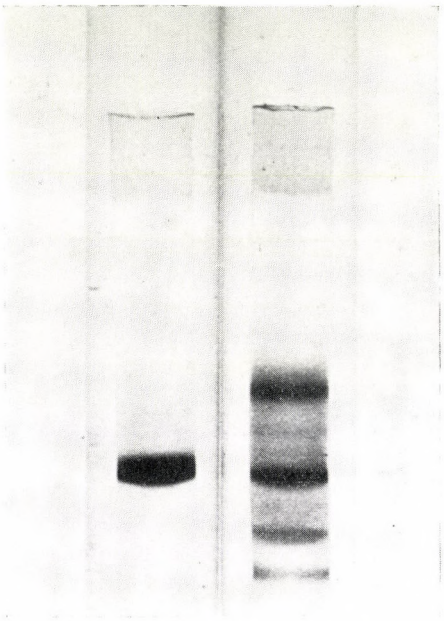

Fig. 2. Purified porcine β-LPH (left) and fraction D′ (right) in polyacrylamide gel electrophoresis at pH 4.5. In contrast to the method of Reisfeld and coworkers (1962) the gel concentration was the same along the whole tube (7.5 per cent). Samples of 50 μg, and 150 μg, respectively, were polymerized into the upper gel. The potential gradient was 30 V/cm, duration of the running was 30 min. Peptides were moving toward the cathode (downwards)

tubes). Tryptophane was determined spectrophotometrically (Beaven, Holiday, 1952). The amino acid composition of the peptide was as follows:

Lys_{10} His_2 Arg_4 Asp_5 Thr_3 Ser_3 Glu_{14} Pro_9 Gly_8 Ala_{14} Val_3 Met_2 $Ileu_1$ Leu_5 Tyr_3 Phe_3 Try_1.

The molecular weight calculated from the amino acid composition only differed by less than 15 per cent from the ultracentrifugal molecular weight.

The corticotropic activity of the peptide as measured by Sayers s.c. test was 0.03 U.

For the measurement of lipolytic activity the retroperitoneal and epididymal fat tissue, resp., of normally fed rabbits and rats was incubated by shaking aerobically in Krebs-Henseleit phosphate buffer at 37° C. The free fatty acid content of the incubated fat pieces was measured after homogenization according to Dole and Meinertz (1960).

Minimal effective doses of β-LPH on the adipose tissue of rabbit and rat were 5×10^{-3} μg and 2×10^{1} μg, resp., as determined from the liberation of free fatty acid in the presence of 100 μg, 10 μg, 1 μg, 0.1 μg and 0.01 μg/ml hormone. Simultaneously, the minimal effective doses of homogeneous corticotropin A_1 were 2×10^{-3} μg and 4×10^{-3} μg, respectively.

On the basis of its chromatographic behaviour, molecular weight, amino acid composition and biological activity the isolated peptide is considered to be β-LPH. A comparison of the amino acid sequence and biological activity of this peptide with that of sheep β-LPH is currently investigated.

We should like to express our sincere thanks to Dr. H. Medzihradszky-Schweiger (Peptide Chemistry Research Group of the Hungarian Academy of Sciences) for the performance of amino acid analyses, and to Dr. P. Závodszky (Institute of Biochemistry of the Hungarian Academy of Sciences) for the ultracentrifugal molecular weight determinations.

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Non-Coordinate Repression of the Synthesis of Enzymes in the Common Part of the Aromatic Amino Acid Biosynthetic Pathway in Escherichia Coli W

(Preliminary Report)

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The first step in the biosynthesis of aromatic amino acids is the formation of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) (Srinivasan, Sprinson, 1959). Three distinct isoenzymes of DAHP synthese (EC 4.1.2.15) were detected in E. coli determined by three unlinked genes (Wallace, Pittard, 1967). All these DAHP synthases catalyze the same reaction but the synthesis of isoenzymes 1a, 1b and 1c is repressed by phenylalanine, tyrosine and tryptophane, respectively (Smith et al., 1962; Doy, Brown, 1965). The formation of chorismate from DAHP, the last intermediate of the common part of the pathway is catalyzed sequentially by six enzymes determined by six unlinked genes (Taylor, Thoman, 1964).

The present paper reports that the synthesis of the first, third and fourth enzymes in the common part of the aromatic amino acid pathway is not repressed coordinately.

Wild type strain of Escherichia coli W and the mutant strains 83-1 (dehydroquinase-less) and 83-24 (shikimate-kinase-less) were kindly provided by Dr. B. D. Davis. The minimal medium A containing 0.2 per cent glucose was used (Davis, Mingioli, 1950). Mutant 83-1 was grown in medium A supplemented with limiting (20–40 μg per ml) or excess (100 μg per ml) shikimate. Mutant 83-24 was grown in the same basal medium supplemented with 0.2 per cent Difco yeast extract, plus 0.2 per cent Sheffield N-Z case and was "released" from repression according to Smith et al. (1962). All cultures were grown at 37° C by shaking on a gyrotory shaker. Growth was followed turbidimetrically by reading the absorbance at 490 mμ. Cultures were harvested and washed with 0.05 M potassium phosphate buffer (pH 7.4). The cells were disrupted by sonication (M.S.E., 60 W). Cell debris was removed and the 40 000 g supernatant was used for determination of the activity of enzymes. Protein was determined by the method of Lowry et al. (1951). For determination of the overall activity of DAHP synthase isoenzymes a standard reaction mixture was used containing 80 μmoles of potassium phosphate buffer (pH 6.4), 5 μmoles of phosphoenol-pyruvate, 5 μmoles of erythrose 4-phosphate and extract in a total volume of 1.0 ml. The mixtures were incubated at 37° C and the reaction was arrested by the addition of 0.4 ml of 10 per cent trichloroacetic acid. The amount of DAHP formed was determined by the thio-

barbituric acid method (Srinivasan, Sprinson, 1959). One unit of enzyme is defined as the amount that catalyzes the formation of one mμmole of DAHP per minute. For determination of the activity of 5-dehydroshikimate reductase (EC 1.1.1.25) a standard reaction mixture was used containing 100 μmoles of Tris-HCl buffer (pH 8.0), 1.5 μmoles of shikimate, 1 μmole of NADP and extract in a total volume

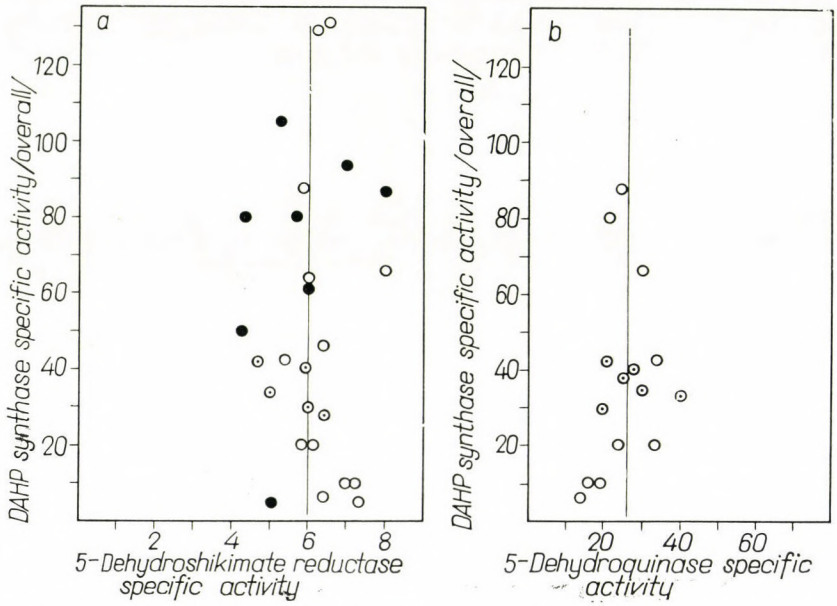

Fig. 1. The ratio of specific activities of some enzymes of the common part of the aromatic pathway at different stages of repression in various extracts. Each point represents the specific activity of two enzymes in an extract of mutant or wild type cells. The wild type extracts are denoted by (⊙), mutant 83-1 by (●) and mutant 83-24 by (○). Specific activity is expressed as units per mg of protein

of 3.0 ml. The reaction was followed by measuring the increase in absorbance at 340 mμ (Yaniv, Gilvarg, 1955). One unit of enzyme is defined as the amount that catalyzes the formation of one mμmole of dehydroshikimate per minute. For determination of the activity of 5-dehydroquinase (EC 4.2.1.10) 5 ml of crude extract containing 70 mg of protein was treated with 0.5 ml of 2 per cent protamine sulphate solution and after centrifugation the clear supernatant was used. The assays were performed in silica cells (light path 1 cm) at 25° C. The cells contained 120 μmoles of potassium phosphate buffer (pH 7.4), 1 μmole of dehydroquinate and the enzyme in a total volume of 3.0 ml. The substrate was added last and the increase in absorbance was read at 240 mμ (Mitsuhashi, Davis, 1954). One unit of enzyme is defined as the amount that catalyzes the formation of one mμmole of dehydroshikimate per minute.

As shown in Fig. 1 the levels of the third and fourth enzymes of the common part of the aromatic amino acid pathway are not regulated, repressed or induced by the end products of the pathway, in contrast to the first enzymes. The level

of the third enzyme, 5-dehydroquinase is 24 units and that of the fourth enzyme, 5-dehydroshikimate reductase, is 6 units per mg of protein. According to our experiments, from the point of view of regulation of the synthesis of enzymes, the common part of the aromatic pathway does not represent an operational unit.

A detailed analysis of the regulation of synthesis of enzymes of the aromatic amino acid pathway is under investigation.

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Ultrastructure of the Striated Muscle and Moiré Patterns

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Evaluating the electron micrographs of the striated muscle a great variety can be observed in relation to the so-called pseudo-striations, and in the recurrence of the thick and thin filaments. These phenomena are interpreted by moiré patterns. Besides the model production of certain phenomena, attention is drawn to possibilities of determining dimensions by the application of a mathematical theory on these patterns.

Introduction

In a previous paper describing investigations along these lines (Achátz, 1967) the fundamental starting idea was that no conclusions can be drawn directly from electron micrographs made of striated muscle in relation of the three dimensional structure. The fact that a two dimensional picture cannot exhibit the characteristics of a three dimensional structure and that the actual structure can be revealed only by the application of different methods, must not be disregarded (Ernst, 1963).

Moreover, the biological material undergoes irreversible transformations in the course of preparation for electron microscopic investigation (fixation, embedding, sectioning, counter-staining, etc.) about which all the stages are not known. The situation in the case of muscle can be complicated further by the fact, that the structural elements which perhaps recur at regular intervals may result in optical artefacts upon microscopic investigation.

Such a micrograph can be seen in Fig. 1. It shows the following three characteristics:

1. Besides striation a so-called pseudo-striation.
2. The periodic change of the thickness of a single filament.
3. The variation of succession of thick and thin filaments.

Several electron micrographs can be found in the literature to illustrate the problem mentioned in point 1, *e.g.* Hodge et al. (1954), Hodge (1955), Hanson and Lowy (1960), Shafiq (1963). The authors do not find a satisfactory explanation for the development of the patterns. In their opinion the patterns are caused by the fact that the sectioned filament layer is not in the plane of section in certain ranges and therefore the section becomes thinner in these places. About the

periodic thickening of the filaments examples can be also found in the literature: Fahrenbach (1964), Brandt et al. (1965), Ernst (1967), Reger and Cooper (1967).

In the following the above mentioned micrographs will be analyzed on the basis of moiré patterns.

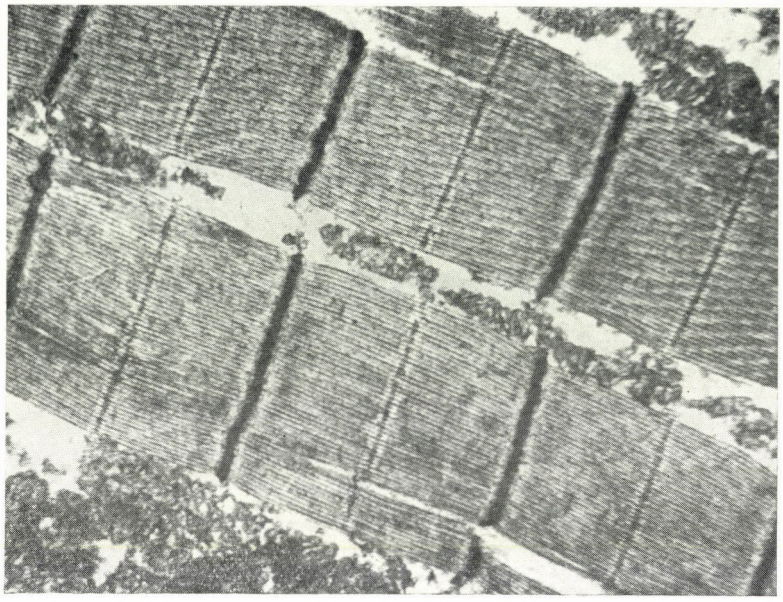

Fig. 1. Longitudinal section of honey bee's wing muscle ×13 800. It shows varied patterns

Method

a) Gratings of a grating constant of 50 μ to 150 μ were produced photographically and two gratings placed upon each other were examined microscopically. The distance between the grating planes was 150 μ. The relatively great depth of focus of the electron microscope was substituted in such a way that an objective of small magnification with a small aperture was applied in the investigations carried out by light microscope. By applying blue light the depth of focus was 0.3 to 0.4 mm.

b) The theory of the moiré patterns was developed by Oster et al. (1964). The superposition of the gratings consisting of equally spaced parallel lines is taken in the present work from the above theory. According to this theory if the grating constant of one grating is a, the other's is b, the lines of the two gratings will enclose an angle of ϑ (Fig. 2) and thus the distance of the created moiré fringes (m) will be

$$d = \frac{ab}{\sqrt{a^2 + b^2 - 2ab\cos\vartheta}} \tag{1}$$

The sinus of the angle of the moiré fringes subtended by the lines of one of the constitutive grating is

$$\sin\phi = \frac{b \sin \vartheta}{\sqrt{a^2 + b^2 - 2ab\cos\vartheta}} \tag{2}$$

If the grating constant of the two gratings is equal ($a = b$) then

$$d = \frac{a}{2\sin\frac{\vartheta}{2}} \sim \frac{a}{\vartheta} \; * \tag{3}$$

in this case $\phi \sim 90°$.

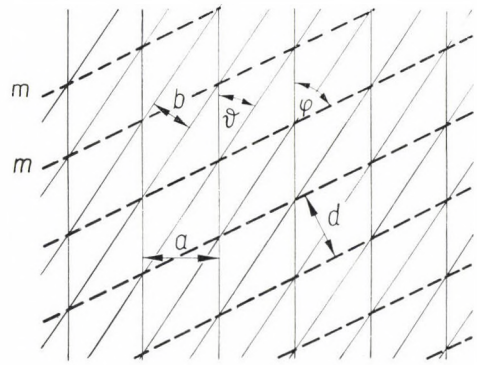

Fig. 2. See in text

In certain cases the lines of the two gratings are parallel to each other ($\vartheta = 0$). In this case the formed moiré fringes are parallel to the lines of the constitutive gratings ($\phi = 0$) and their distance is

$$d = \frac{ab}{|a - b|} \; ** \tag{4}$$

If the ultrathin longitudinal muscle section (300 to 600 Å thick) is interpreted as two parallel grating planes then the microscopic photograph of the parallel grating planes of our models can be related to the mentioned muscle structure as well.

Parallel grating planes can be dealt with in general terms because in the case of parallel projective beam of rays when they fall perpendicularly on this

* $d = \dfrac{a^2}{\sqrt{a^2 + a^2 - 2a^2\cos\vartheta}} = \dfrac{a^2}{a\sqrt{2 - 2\cos\vartheta}} = \dfrac{a}{2\sqrt{\dfrac{1 - \cos\vartheta}{2}}} = \dfrac{a}{2\sin\dfrac{\vartheta}{2}}$

** $d = \dfrac{ab}{\sqrt{a^2 + b^2 - 2ab}} = \dfrac{ab}{|a - b|}$

projective plane, the divergence from the parallel planes can be traced back to the parallel planes (Fig. 3). The new grating constant will be:

$$d' = d \cos \alpha$$

Since the aperture of the incident beam of light in the electron microscope is 10^{-3} to 10^{-4} radian it is in good agreement with the parallel beam of rays.

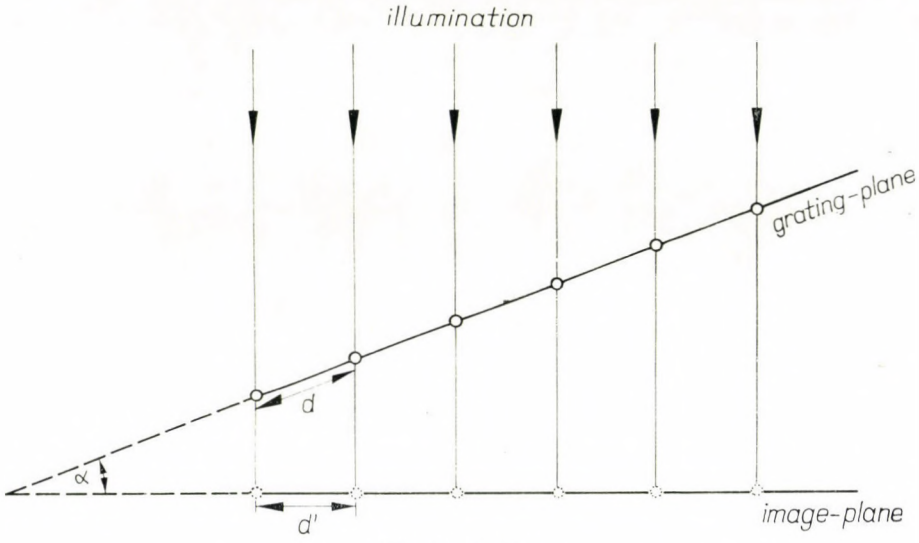

Fig. 3. See in text

Results

1. In Figs 4 *a*, *b*, and *c* such moiré fringes are produced with two gratings which have equal grating constants, $a = b = 1$ mm. The angle enclosed by the

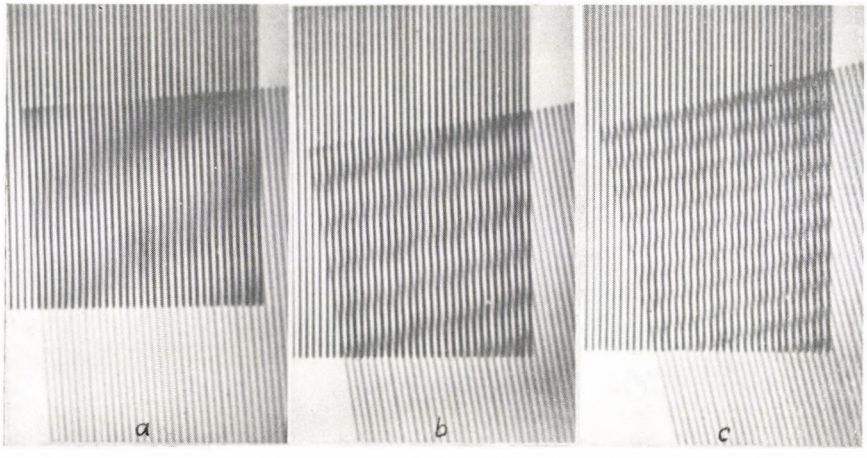

Fig. 4. Moiré pattern in the case of gratings localized in two parallel planes

two gratings in Fig. 4a is $\vartheta = 4°$ and according to equation (3) $d = 14.3$ mm; in Fig. 4b $\vartheta = 9° 50'$ accordingly $d = 5.8$ mm; in Fig. 4c $\vartheta = 15° 20'$, the distance of the moiré fringes is $d = 3.7$ mm. Because the grating constant of the two gratings is equal the moiré fringes are approximately perpendicular to one of the constitutive gratings in these figures. Fig. 5 is also the superposition of two gratings, but here both the two grating constants and the thickness of the lines are different. The grating constants are: $a = 1.2$ mm, $b = 1.7$ mm. The angle enclosed by the lines of gratings is $\vartheta = 9° 15'$. The distance of moiré fringes is $d = 3.7$ mm, from eq. (1). The moiré fringes subtend a $\phi = 20° 20'$, angle by gratings of a grating constant of 1.2 mm.

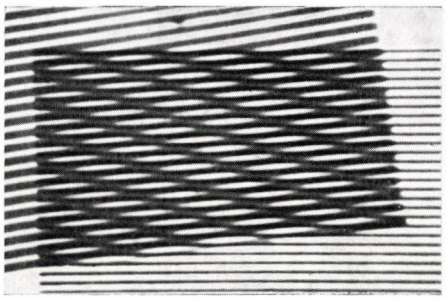

Fig. 5. Moiré patterns formed by gratings of different grating constants

Should the angle enclosed by the gratings change, or if the lines of gratings are not straight, the moiré fringes will not be straight either but will show varied patterns. This is demonstrated in Fig. 6 in the electron micrograph of a longi-

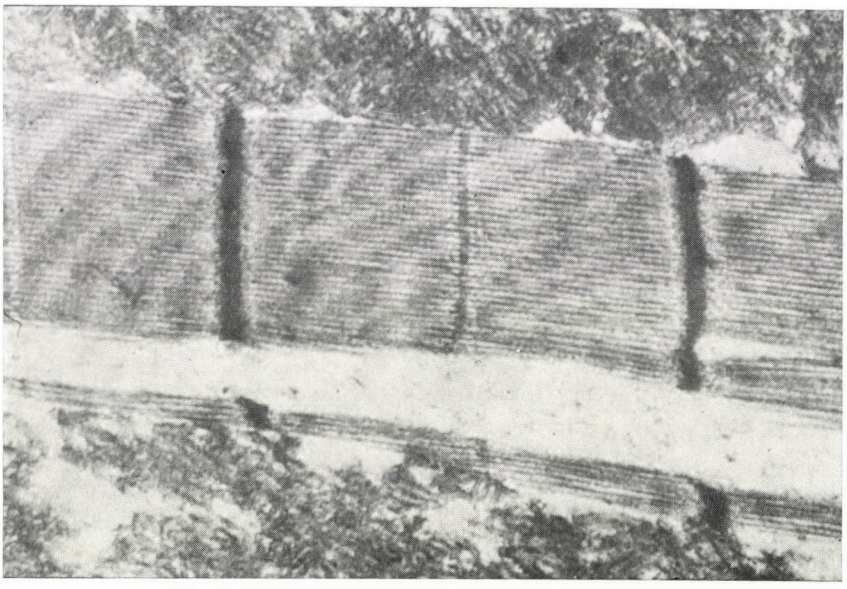

Fig. 6. Longitudinal section of honey bee's wing muscle $\times 19\,200$. Moiré patterned

tudinal muscle section. Unfortunately the quantitative evaluation has to be given up in such a complicated case.

2. The situation is even more complicated in the case of micrographs where the single filaments periodically become thicker and thinner (Fig. 7). In Figs 8 and 9 such moiré patterns are shown as a model-like analogy the character of which is essentially influenced by the fact that of the two imaged grating planes one does not fall into the sharply focussed interval of the microscope. In Fig. 8

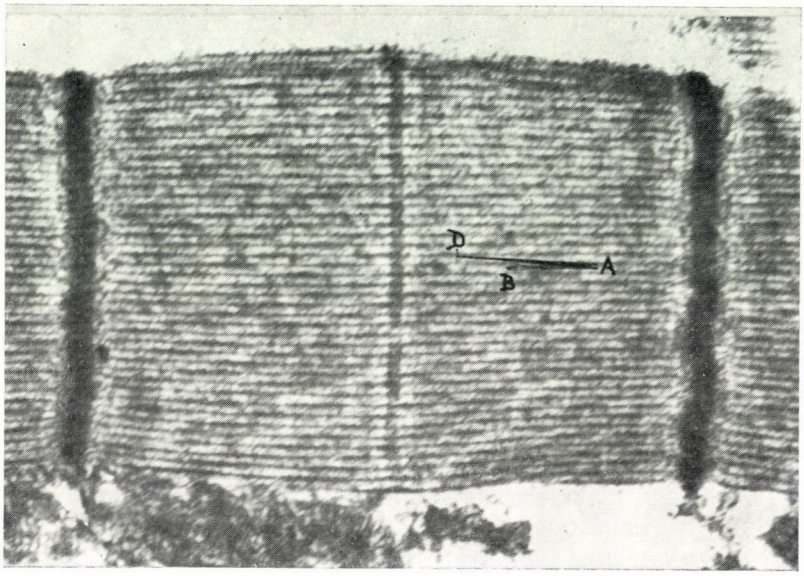

Fig. 7. Longitudinal section of honey bee's wing muscle ×31 200. It shows the periodical change of thickness of the filaments. The measurable AB and AD distances are shown in the appropriate section

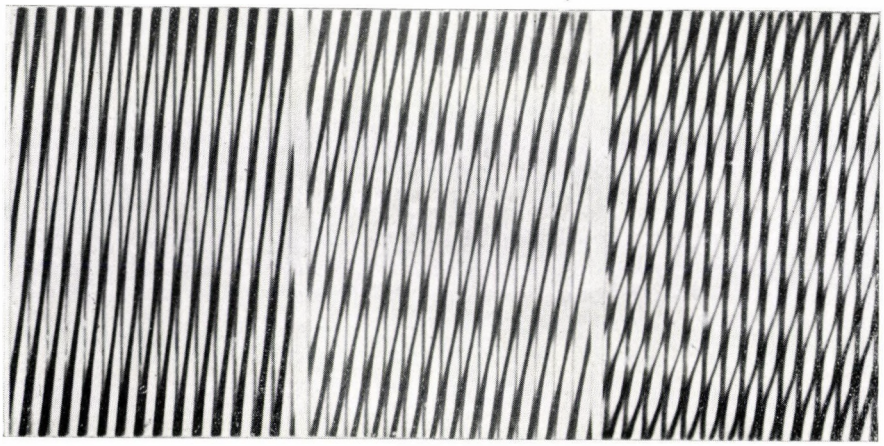

Fig. 8. Moiré pattern in the case when both grating planes are well focussed by the microscope

both planes are well focussed, in Fig. 9 only one of them. The mechanism of formation of these patterns can be well seen in the Figures.

In the following it will be shown that these patterns are also suitable for quantitative evaluation. In Fig. 10 a magnified part of Fig. 8 is shown. The thickenings are produced at the intersections of the lines of the grating. The four adjacent thickenings form a parallelogram, the peaks of which are designated as *A*, *B*, *C*, and *D*. The distance *AB* and *AD* are well measurable. If the grating constant of one grate is *a*, that of the other (which is invisible) is *b*, then

$$b = \frac{AB}{AD} \cdot a \tag{5}$$

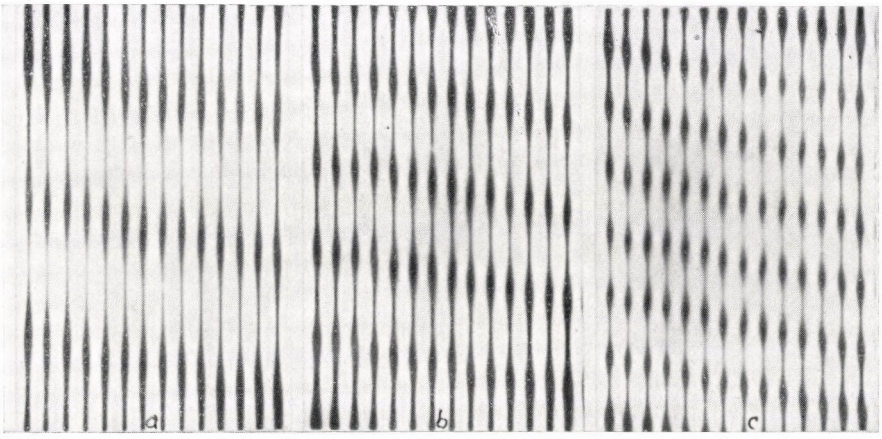

Fig. 9. Moiré pattern, in the case when only one of the grating planes is well focussed

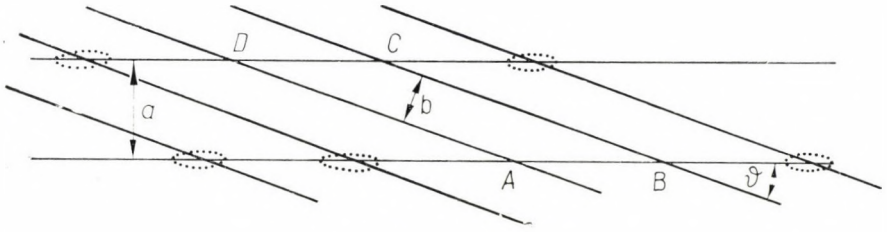

Fig. 10. See in text

The grating constant of the invisible grating can be calculated from the grating constant of the visible grating and from the distance of thickenings (AB and AD). In Figs 9a, 9b, and 9c AB = AD and $a = 2.5$ mm, then according to equation (5) $b = 2.5$ mm. Accordingly, these patterns are produced by certain turning of angle of two gratings having equal grating constants. This can be controlled in Fig. 8. It can be seen that by increasing the angle enclosed by the lines of the two gratings, the thickenings will shorten and their closeness will increase.

Fig. 11 shows a similar model, here $a = 1.8$ mm, AB = 9.2 mm, AD = = 15 mm, consequently the grating constant of the "invisible grating", according to the calculation, is

$$b = \frac{9.2}{15} 1.8 = 1.1\text{mm}$$

in agreement with the real value.

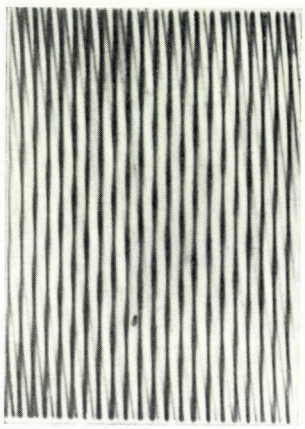

Fig. 11. Moiré pattern in the case of two gratings of different grating constants. Only one of the two grating planes is well focussed

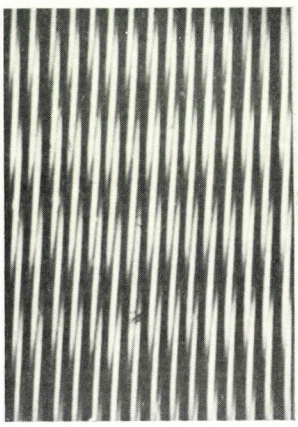

Fig. 12. Moiré patterns formed by gratings of equal grating constants and of different thicknesses of lines. Both grating planes are well focussed

3. In models demonstrated in Figs 4, 8, and 9, the thickness of lines of both gratings was equal. It will be demonstrated that the essential data of the formed patterns do not depend on the thickness of the lines of gratings but on their grating constants. In Fig. 12 both of the gratings are visible, their grating constants are equal, i.e. $a = b = 2.5$ mm, the thickness of lines is visibly different. In Fig. 13, where only one of the gratings is well focussed, AB = AD = 13 mm, and $a =$ = 2.5 mm, therefore $b = 2.5$ mm according to equation (5). The corresponding data of the electron micrograph of a muscle section in Fig. 7 are: The distance of the visible filaments $a = 1.3$ mm which equals 430 Å; AB distance is 11.5 mm, AD distance is 18 mm, thus according to equation (5), $b = 0.83$ mm, which equals 270 Å. It is well discernible that the periodic thickenings are continuing in the *H-zone and M-line*, as well.

4. If two gratings are placed upon each other in such a manner that their lines are parallel, the formed moiré fringes will be parallel to the lines of the grating, too. It seems possible that the variability of systems consisting of thick and thin filaments is due to similar phenomena.

One of the model types is shown in Fig. 14. *Two exactly equal gratings, where the grating constants and the thickness of lines are also the same*, are placed upon each other in such a manner that the lines of one grating should fall precisely into the line spaces of the other. The lines of the lower grating seem to be thinner by microscopical investigation. For comparison, an electron micrograph of a muscle section can be seen in Fig. 15, in which the thick and thin filaments are well observable. The densitometric diagram of the two former

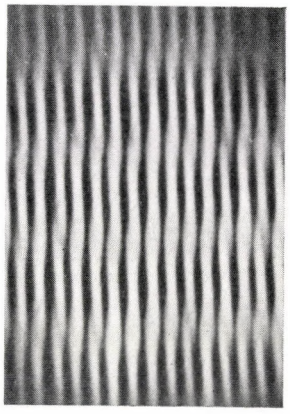

Fig. 13. Same model as in Fig. 12, but only one of the grating planes is well focussed

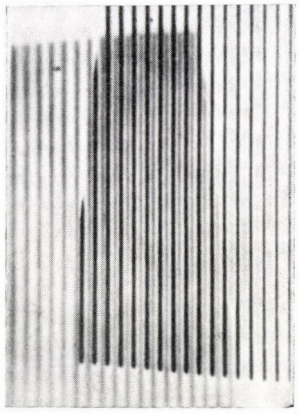

Fig. 14. Gratings of equal grating constant and thickness of line. The lines of the lower grating seem to be thinner

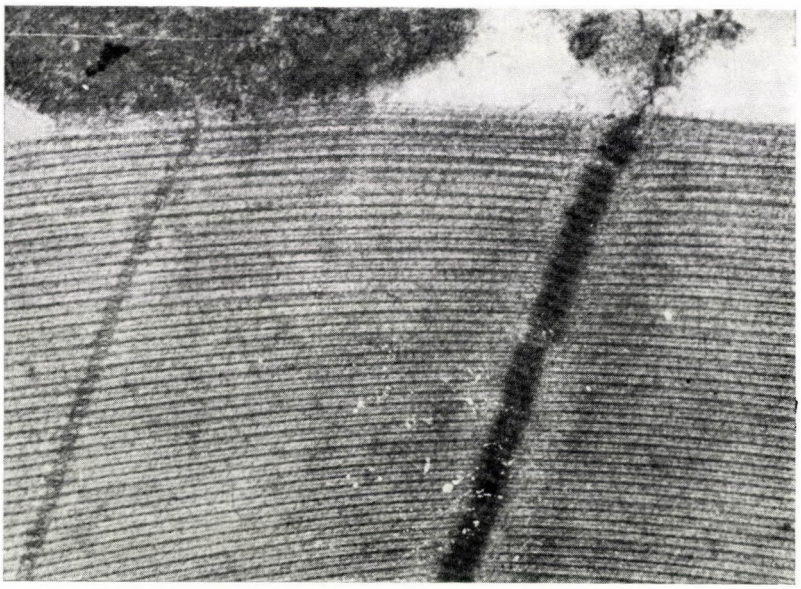

Fig. 15. Longitudinal section of honey bee's wing muscle ×41 600

figures can be seen in Fig. 16: Fig. 16a is a diagram taken of the model (Fig. 14), and Fig. 16b is a diagram taken of muscle (Fig. 15).

If such two gratings of parallel lines are placed upon each other where the rate of their grating constants is 3 : 2, regularly alternating thick and thin lines are gained in this case, too. This is shown in Figs 17 and 18. In Fig. 17 the

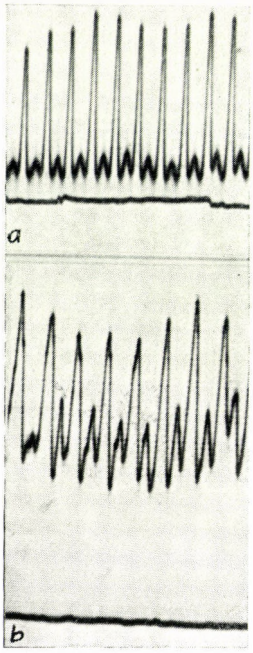

Fig. 16. a: Densitometric diagram of Fig. 14; b: Densitometric diagram of Fig.15

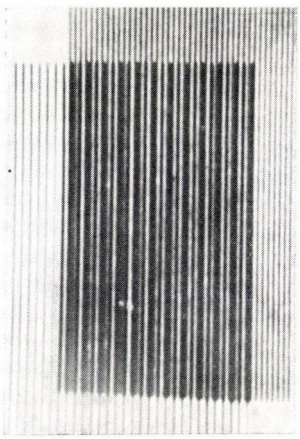

Fig. 17. Formation of thick and thin lines with two gratings of different grating constants and of equal thick lines

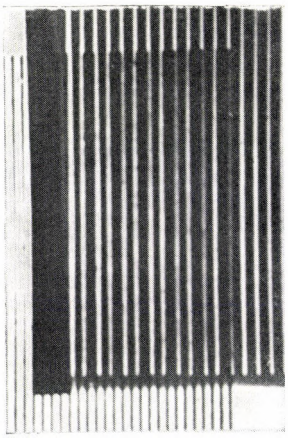

Fig. 18. Formation of thick and thin lines with two gratings of different grating constants and of different thick lines

thickness of lines of both gratings is equal, and the grating constants are $a =$ $= 1.05$ mm, $b = 0.7$ mm. In Fig. 18 the thickness of lines of the gratings is also different, the value of the grating constants are: $a = 1.77$ mm, $b = 1.18$ mm.

Discussion

In paragraphs 1, 2, and 3 describing the results, models were demonstrated in which the lines of gratings enclose an angle with each other. To the question how this turning comes into being in the case of two layers of filaments, no answer can be given yet.

Owing to the fact that thickenings of the filaments can be discovered in the H-zone and M-line as well (Fig. 7), the conclusion can be drawn that the two filaments causing the thickening are continuous through the whole sarcomere.

Models demonstrated in paragraph 4 show that the periodically alternating thick and thin filaments can be the result of the superposition of two equal gratings. An interesting observation can be made in Figs 14 and 18: if the lines of a grating run into another grating system, it seems as if their thickness had changed.

The results show that the evaluation of muscle electron micrographs with the help of moiré patterns offers a possibility for a better understanding of the geometrical composition of the muscle structure. At the same time new problems are raised which have to be solved.

Acknowledgement

The author is indebted to Professor E. Ernst for his advice in the course of the experiments.

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Mechanical Properties of the Flight Muscle of the Bee, I

Resting Elasticity and Its Ultrastructural Interpretation

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The length—tension diagram of the resting bee muscle can be divided into two phases. Up to about 100 per cent elongation the tension steeply increases. From this length on up to about 200 per cent the tension exhibits a slight increase only. The resting elasticity is attributed to the set of the C-filaments.

Introduction

At extreme degrees of passive stretch the two different kinds of filaments (Huxley, 1953; Hanson, Huxley, 1955) cease to overlap each other (Carlsen et al., 1961; Sjöstrand, 1962; Page, 1965; Carlsen et al., 1965; 1965a; Hoyle, 1968). The sarcomere length which corresponds to the end of the overlap of the myosin and actin filaments is called *no overlap point*. According to the team of A. F. Huxley the tetanic tension falls practically to zero at fiber lengths corresponding to this sarcomere length (Huxley, Peachey, 1961; Gordon et al., 1964; see also Podolsky, 1964).

Insect flight muscle is, in general, less extensible (Pringle, 1967). Far below the no overlap point irreversible stretch zones (Hanson, 1956; White, 1966) appear due to the breaking away of the actin filaments from the Z-lines. This breaking away was clearly demonstrated by the electronmicrographs of White (personal communication). In contrast, the flight muscle of the bee can be stretched over 300 per cent of its resting length offering, thus, the possibility of being studied in a wide range of lengths within which the change in the degree of overlap cannot play any role.

Our earlier observations (Garamvölgyi, 1965; 1966; 1966a) have shown that until a sarcomere length came rather close to the estimated no overlap point the structural changes of the myofibril were in agreement with those established by Hanson and Huxley (1955), but from this critical sarcomere length on the A-bands began to stretch. The structural expression of the elongation of the A-band is the appearance of zones of decreased density and birefringence at the A—I junctions and later also in the middle of the A-bands. We designated these zones E- (elongation) zones. On the basis of our experience we proposed a sarcomere model for this kind of muscle (Garamvölgyi, 1965) which model included a second kind of thin filaments (Auber, Couteaux, 1962; 1963; Garam-

völgyi, 1963) representing mechanical connection between the end of the myosin filaments and the Z-line. These filaments have recently been designated as C-filaments by Pringle (1967).

Materials and Methods

For the measurements we used bees. Their head, abdomen and prothorax – together with the first pair of legs – have been removed. The surviving two pairs of legs as well as the wings have been bound together near their origin by means of a 0.1 mm flexible copper wire (Fig. 1). Subsequently the lateral chitin plates (pleurits) were carefully cut through with a blade. One of the dorsoventral muscles was entirely cut off and the peripheral fibres on the other side were also interrupted. Thus only a unilateral bundle of fibres lying the closest to the sagittal plain was preserved.

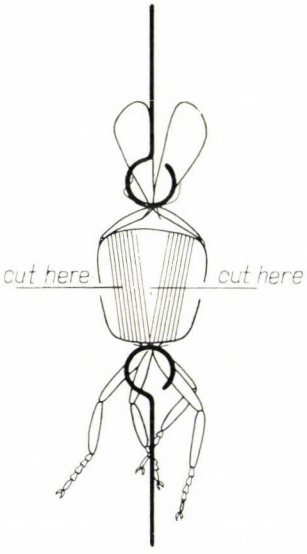

Fig. 1. Schematic representation of a transversal section through the mesothorax of a bee demonstrating the preparation of the muscle bundle

The muscles have been stretched by the "Gleitmikromanipulator" (gliding micromanipulator) of Zeiss-Jena (Fig. 2). The muscles were strongly bound to steel hooks (1) by means of the wire. The one hook was rigidly attached to the mowing brass plate of a capacity transducer (2) held by the right stative of the micromanipulator fixed at its terminal position. The other hook could be moved together with the gliding sled of the left side stative. The movement of the sled was limted by a micrometer screw (3) with a nonius-scale and a rotating drum which enabled a displacement control in steps of 0.01 mm. By rotating the screw the sled was released and it could glide backwards drawn by a counterbalance (4)

coupled with the gliding sled through a pulley (5). The controlled gliding range of the sled was 6.5 mm.

The specimens were immersed into a plexiglass vessel containing a physiological solution (Pringle's solution). Fine slits on both sides of the vessel permitted the penetration of the hooks. The resting length of the muscles has been roughly determined as the length at which resting elasticity just became perceptible. This length was adjusted before each experiment by the coarse transverse movement control of the micromanipulator, without moving the gliding sled.

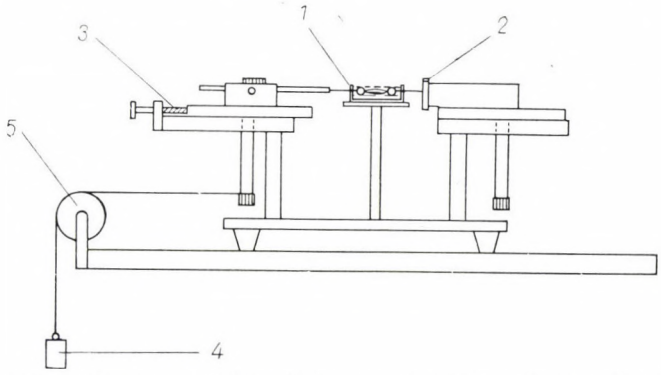

Fig. 2. Schematic representation of the experimental equipment. See text

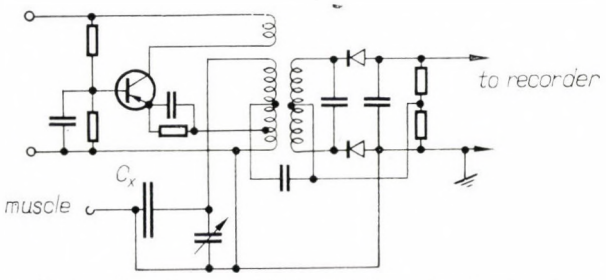

Fig. 3. Connection diagram of the mechanoelectric transducer

The capacity transducer (Mórocz-Juhász, Örkényi, 1967) of 5 pF consisted of two brass plates. By bending the outside plate – which is proportional to the tension exerted by the muscle – the mechanical variable was transformed into an electrical one (Fig. 3). By the stretch of the muscle the capacity of the condenser C_x decreases, and a voltage change proportional to the tension arises at the output. The sensitivity of the transducer is 5 mV/pond, and its linearity better than 5 per cent. The d.c. output was connected to the ink recorder "Micrograph" BD 2 of Kipp-Delft. The recording was continuous, with a chart speed of 600 mm/h. In one part of the experiments an RCA 5734 tension transducer was used. The records differed from those taken with the capacity transducer in no respect.

Glycerol extraction of the muscles was performed according to the schedule of Huxley (1963). As relaxing medium we used a slightly modified form (Chaplain et al., 1965) of the ATP-EGTA-KCl-$MgCl_2$ solution described by Pringle (1967). The glycerol-extracted muscles were stored in a deep-temperature refrigerator for at least 3 weeks.

Results

a) *Transient and equilibrium tension*

The experiments have been performed by employing successive stretches of 0.5 mm (Fig. 4). In each case we observed a sudden rise of tension immediately followed by the well-known phenomenon of "stress-relaxation", a decrease of

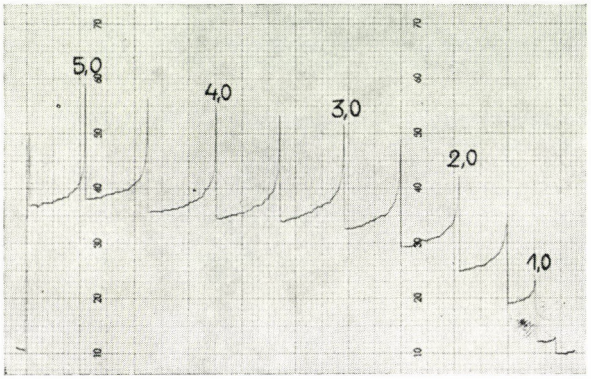

Fig. 4. Record of an experiment performed on fresh muscle (starting from the right). The numbers above each second peak represent the elongation in mm

tension in form of an exponential curve tending to an equilibrium level (see also White, 1966; Pringle, 1967). In the present paper we are only dealing with the "equilibrium tension", i.e. with the static tension values reached after each stress-relaxation. Here, we only mention that the transient tension rise obviously increases within the first 2 or 3 mm range of elongation. From about this length on the height of the peaks exhibits only an insignificant increase, sometimes even a slight decrease.

With respect to the equilibrium tension we observed that within the range up to $\Delta l = 2.5$ mm the tension steeply increased. Beyond this length the equilibrium tension remained rather constant. The muscles usually could stretch until $\Delta l = 5-5.5$ mm, at which length they teared. Since the *in situ* length of the fibre bundles is close to 2.5 mm, the maximum elongation corresponds to about 200 per cent. The length corresponding to $\Delta l = 2.5$ mm is therefore nearly equal with 100 per cent elongation.

b) *Statistical evaluation*

The records have been subjected to a simple mathematical analysis. Due to the fact that the elastic properties of the muscles consistently exhibited an

abrupt change at about 100 per cent elongation the tension values obtained at $\Delta l = 2.5$ mm were taken as unit and the tension values belonging to the different length changes were compared with this tension in each experiment. The introduction of relative tension values was necessary because of the differences in the thickness of individual fibre bundles.

If we assume that the length–tension curve can be divided into two different phases two regression lines would fit the measured tension values (Fig. 5). The first regression line calculated in the interval $0 \leq \Delta l \leq 2.5$ mm has the analytical form:

$$P = 0.40\, \Delta l + 0.02$$

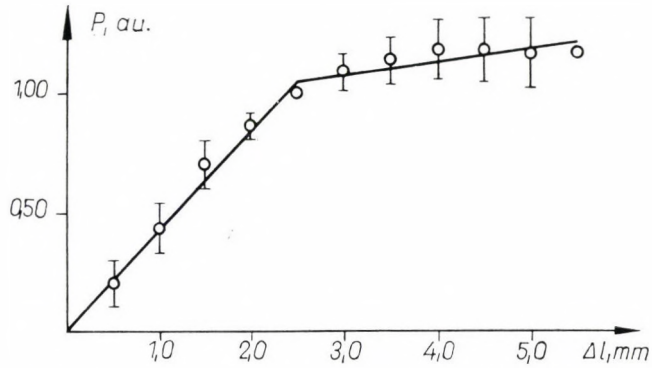

Fig. 5. Statistical length–tension diagram constructed from the values of 20 experiments. See text

where Δl is the elongation in mm measured by the micrometer screw and P is the tension in terms of arbitrary units.

The second regression line valid in the interval 2.5 mm $\leq \Delta l \leq$ 5.5 mm has the formula:

$$P = 0.06\, \Delta l + 0.91$$

The mean values of tension in arbitrary units and their standard deviations are plotted against elongation in Fig. 5, which shows the two regression lines calculated. It can be seen that within the range up to about 100 per cent elongation, the increase of the equilibrium tension is rather linear and the increase of tension corresponding to an elongation of 1 mm nearly equals 40 per cent of the tension developed at 100 per cent elongation. From this length on the muscle behaves like a more extensible body since the regression coefficient is with an order of magnitude smaller than that calculated for the first range of elongation.

c) *Glycerol-extracted muscles*

We performed also a series of preliminary experiments on glycerol-extracted muscles in the presence of an ATP-EGTA-Mg^{++}-containing relaxing solution (Chaplain et al., 1965). Before the experiments the muscles have been washed

in the relaxing solution for several hours. The stretch of the muscles was carried out in this solution just in the same way as described for fresh muscles (Fig. 6). Neither in the range of possible elongation, nor in the changes of tension following each individual stretch (transient tension, stress-relaxation and development of equilibrium tension) was there any difference to be found when compared with fresh muscles.

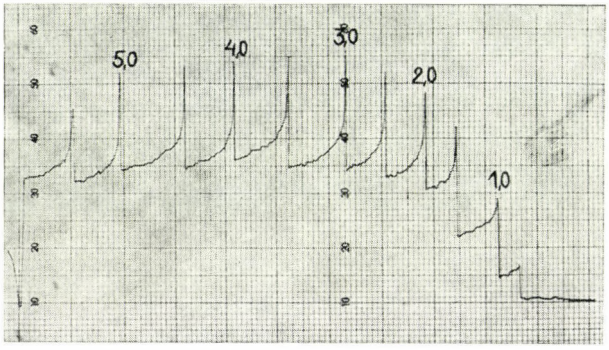

Fig. 6. Record of an experiment performed on glycerol-extracted muscle in a relaxing medium (starting from the right). Compare with Fig. 4

d) *The possible sources of error*

The drastic errors which could introduce serious misinterpretations could be eliminated relatively easily. The loosening of the knots serving for the fixation of the specimen could usually be recognized after the first few elongations (insignificant increase of tension, lack of stress-relaxation). The tearing of whole fibres could be recognized by a sudden fall of tension mainly occurring during the initial rapid phase of the stress-relaxation.

Although chitin is a highly inextensible material the wings and the upper legs of the bees could perhaps contribute to the elongation if even to a not very considerable extent. Because, however, muscle is much more extensible than chitin we regarded this effect negligible.

A source of error can originate in the difficulties of the exact determination of the resting length. This is the consequence of the small size of the specimen and also of the fact that this kind of muscle has an extremely short physiological working range and thus the resting length must be close to the minimum in situ length. This source of error might probably find its expression in the fact that the regression line (Fig. 5) does not start exactly with $P = 0$.

Further error could be introduced by the different lengths of the fibres of the same bundles. In the case of thick bundles this resulted in a lack of an abrupt change of elasticity at 2.5 mm elongation. We intended to minimize this effect by preserving thin bundles only, consisting of the most medial fibres.

It is well known that insect muscles have neither myotendinal junctions nor sarcolemmal sheets. The myofibrils are directly attached to the chitin substance

(Auber, 1963). As a consequence, the possible error introduced by the connective tissue is negligible and the resting elasticity must be attributed virtually to the myofibrillar substance alone (see also Pringle, 1967).

Discussion

Numerous works of this Institute dealt with the relationship between structure and mechanical properties (Ernst, 1963; Kerner, 1966). It is interesting that the statistical length–tension diagram (Fig. 5) has a form closely resembling the "I-band length–sarcomere length" curve of bee myofibrils (Garamvölgyi, 1966). This resemblance suggests a relationship to exist between the striation pattern and the mechanical behaviour of the muscle substance, even if the sarcomeres alongside a fibre are not exactly of the same length.

The sarcomeres of the insect muscle are virtually occupied by the A-band. The constant length of the A-band (below 5 μ sarcomere length) is 2.7 μ in the case of the bee (Garamvölgyi, 1966). Since we never observed a "double overlap" of the actin filaments (Huxley, 1963) – assuming the constant length of both kinds of filaments (Page, Huxley, 1963) – the length of an I-segment (+Z-line) cannot be over 2.8 μ. The theoretical no overlap point of the bee flight muscle myofibrils is, thus, equal with 5.5 μ. As mentioned above the length of the A-band+ the width of the Z-line must be very close to the sarcomere length at rest and we do not commit a serious fault, if we assume 2.8 μ as being the sarcomere length at resting length. It follows from these values that the estimated no overlap point roughly corresponds to an elongation of 100 per cent.

Our earlier results have shown that up to a sarcomere length rather close to the theoretical no overlap point the length of the I-band increased parallelly with the decrease of the degree of overlap (Garamvölgyi, 1965). At this length ($S = 5\ \mu$) a structural barrier seems to prevent any significant further elongation of the I-band and the further increase of the sarcomere length is due to the eongation of the primary filaments (Garamvölgyi, 1965; 1966; 1966a). Since bllow 100 per cent elongation the majority of the sarcomeres has to be within the range of the changing degree of the overlap and, furthermore, over 100 per cent elongation at least a considerable body of the sarcomeres has to pass the no overlap point, we assume the first steep phase of the length–tension curve to correspond to the range of the sliding movement and the second – flat – phase to be mainly determined by the elongation of the myosin filaments. Since the elongation of the A-band begins slightly below the no overlap point (at 5 μ instead of 5.5 μ) it might be assumed that a short zone of overlap still persists (Fig. 7).

It could formally be supposed that the last few interaction sites of the remaining narrow overlap zones could be responsible for the elongation of the A-band beyond 200 per cent of resting length. Notwithstanding, the resting elasticity can hardly be interpreted by means of actin-myosin interaction, since the tension bears a reverse correlation to the number of the possible reaction sites and the tension reaches its almost maximum value just when the number of the reaction sites reached its minimum.

In our experiments performed on glycerol-extracted muscles put into a relaxing medium we intended to detach the eventual myosin-actin links. Since we failed to establish any difference between the behaviour of the fresh muscles on the one hand and of the glycerol-extracted relaxed muscles on the other, we assume that the myosin–actin interaction does not contribute to the resting elasticity, not even within the range of the sliding movement.

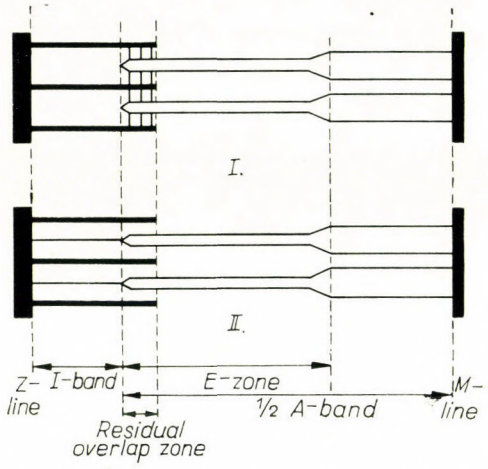

Fig. 7. Diagrammatic representation of the two possible reasons for the elongation of the myosin filaments. Top (I): the last few interaction sites, bottom (II): C-filaments

Our sarcomere model (Garamvölgyi, 1965) seems suitable for describing the mechanical properties of the resting bee muscle. On the basis of our observations we supposed the Z-lines to contain the coiled substance of the C-filaments (Garamvölgyi, 1967, 1968). In the case of the passive stretch the C-material might be drawn out of the Z-lines till the total uncoiling of the C-filaments (Fig. 8). The uncoiling of the C-filaments occurs simultaneously with the decrease of the overlap by the relative sliding movement resulting in the increase of the length of the I-bands. Thus the first steep part of the length-tension diagram (Fig. 5) can find its explanation in the uncoiling of the C-filaments.

In their totally uncoiled state the relatively inextensible C-filaments might prevent the further increase of the length of the I-band. From this length on the myosin filaments begin to stretch under the influence of the tension exerted by the C-filaments on their both ends. This would find its expression in the second, nearly constant range of the length-tension diagram. Consequently, the striking resemblance of the length-tension diagram to the "I-band length–sarcomere length" diagram would not be merely accidental, both diagrams being determined by the C-filaments probably responsible for the resting elasticity of the muscle substance.

In this connection recently Pringle (1967) raised the possibility of the participation of the C-filaments in the development of tension in the resting insect

muscle. Our present findings are in agreement with this suggestion. The differences between our results and those of the Pringle's team might have their basis in the different length of the C-filaments. The length–tension diagram obtained by White (1966) on waterbug (Lethoceros sp.) muscle extends to the range up to about 15 per cent elongation and its second flat phase begins at 9 per cent. In the same material Ashhurst (1967) failed to find C-filaments by the electron microscope. This apparent lack of C-filaments in this insect can also be explained by shorter C-filaments, i.e. by a smaller amount of the C-material.

The authors thank Mrs. Mary Cser-Schultz for her skilful technical assistance.

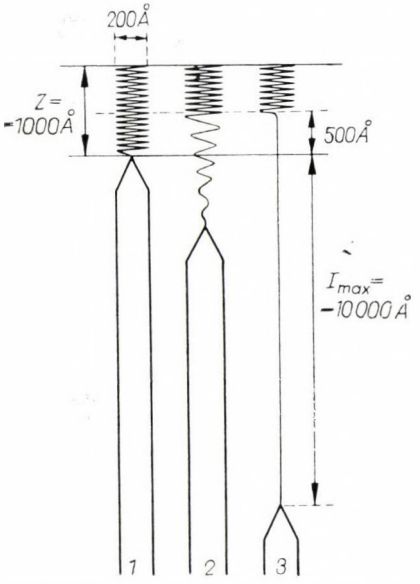

Fig. 8. Diagrammatic representation of the possible contribution of the C-filaments to the first phase of passive stretch. The actin filaments are not represented. (From Garamvölgyi, 1968)

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Experiments on the Hardness of Bone by Vickers Microhardness Measurements

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Authors have studied the hardness of bones by Vickers microhardness measurements. Between the values of hardness of the corticalis and of spongiosa 30 per cent difference was observed. This difference may be of great importance from the point of view of incorporation of the transplanted bone. The fluctuations of values observed on the peripheral part of the corticalis support the suggestions reported till now about the uneven mechanical characteristic of the corticalis. No considerable difference was perceptible between the microhardness of human and bovine bones. The mechanical analogy of bone and copper can be confirmed on the basis of the experimental results obtained.

Introduction

Carlström performed the examination of the hardness of bone by microhardness measurements the first time in 1954 to elucidate the eventual correlation between mineralization and the data obtained by autoradiography. According to his conclusion, mineralization is directly proportional to microhardness. Engström in 1956, Amprino in 1958 and 1961, and Rosate in 1958 studied the correlation between the microhardness and the different physical characteristics of the bone. Palma and co-workers in 1965 on the occasion of measurements performed on compacta observed an increase in microhardness while comparing the intercalary lamina and lamina specialis. Weaver in 1966 examined the microhardness of bone in some bone diseases and found that microhardness was the exact and reliable index of mineralization of bones.

Concerning the measurements of microhardness performed on bones till now, the following should be pointed out: 1) By sclerometers only comparative but no absolute values can be gained. 2) It is absolutely necessary that measurements be performed with identical instruments and identical methods. 3) The results obtained depend on the kind of bone, namely on the degree of moisture-content of the bone.

The investigations of the above-mentioned authors cover the relations of the microhardness of corticalis of bone. Only Weaver characterizes the spongiosa in such terms without furnishing numerical data. In the course of our own investigations,

1) Our aim was to gain data about the difference of microhardness of corticalis and spongiosa.

2) On the basis of the measurement of microhardness we re-examined the known analogy between bone and copper.

Principles in measurements and technology

Hardness is one of the physical characteristics of substances of solid state. Hardness of the substance is measured by the resistance which is exerted against the entering of another substance. Accordingly the functional principles of sclerometers in use are the following: An instrument of puncture of a given execution is pressed into the surface of the substance under investigation with definite force and the effect of impressure is measured (surface, deepness, width of engraving). The substance of the instrument of puncture is chosen to be harder than that of the substance to be examined (hard metal or diamond). The deeper the instrument of puncture penetrates into the surface of the substance the softer is the examined substance.

The instrument of puncture in Vickers' examination of hardness was a diamond pyramid where the angle of inclination of the two opposite surfaces was 136°. Hardness is supplied by the quotient of the loading force, and the surface of impressure, namely

$$HV = \frac{P}{F} \text{ kp/mm}^2$$

Owing to the fact that it is difficult to determine the surface of impressure directly, the surface is calculated from the diameter of the surface. On the basis of diameter d of the surface hardness is calculated by the following equation:

$$HV = 1.854 \frac{P}{d^2} \text{ kp/mm}^2$$

When P loading force is chosen to be large, great impressure is gained depending on the hardness of the substance, and thus the average hardness can be determined. Smaller impressure is gained by decreasing the loading. Under the circumstances when loading is a few ponds, the hardness of the individual parts can be measured separately. Vickers hardness measured by such small loading (smaller than 0.5 kp) is the so-called Vickers microhardness.

The measurements of microhardness were performed by Hanemann's microhardness tester. The instrument functions on Vickers' principle. The diamond pyramid was mounted in the objective of a microscope. The holder of the center punch was supported by a spring and thus when the object was pressed against the diamond pyramid the loading force was given by the pressure of the spring. The pressure of the spring can be determined by a scale mounted in the micro-sclerometer. The diameter of the impressure is measured by ocular micrometer.

Before measurements the Hanemann's micro-sclerometer must be calibrated. First of all the value of the divisions of the scale is determined. This is performed as follows: a suitable weight is placed on the center punch of the sclerometer and is examined how far the scale has moved. In our case under the effect of 5 p weight the displacement was 2.5 divisions and under the effect of 15 p weight the displacement was 7 divisions. The characteristic of the spring is nearly linear and thus on the basis of the correlation it can be determined that the 10 divisions used in the measurement equals 22.5 p loading force.

After that the enlargement of the micro-sclerometer is determined and thus the real value of the diameter of the impressure can be calculated from the diameter measured by the ocular micrometer. The object micrometer is put in the place of the objective on the objective table and thus the adequately magnified picture of the scale is seen through the objective of the sclerometer and through the ocular micrometer, and consequently it can be determined that in reality how many millimeters equal one division of the ocular micrometer (which can be divided to further 100 divisions by the micrometer head). According to the calibration 1.00 division of the ocular micrometer corresponds to 0.03 mm, that is to 30 μm.

The examination of hardness was performed by a metal microscope of Zeiss Neophot type. The metal microscope, as it is known, functions with reflecting light. The object, in the present case the bone, must be prepared for the examination. The preparation consists of polishing and glazing (burnishing). The pieces to be examined were embedded into plastic for preparation.

Experimental substances

The examined bones were in every case conserved bones supplied by the Department of Tissue Conservation of the National Traumatological Institute.* Partly desantigenizated calf tibia and partly deep frozen human tibia were measured. The bones were taken from the shin-bone of 30 to 45 years old casualty victims, on the one hand, and from the tibia of 12 months old animals, on the other. Examinations were performed at room temperature after standardized conservation. Taking into consideration that the change in some physical characteristics (moisture, etc.) is inevitable during the course of mechanical preparation and embedding into plastic, but the change taking place is always exactly similar, and that the comparison of spongiosa and corticalis was performed on the same bone, different kinds of standardization were disregarded during the experiments. The bone to be tested was cut perpendicularly to the axis of the bone while preparing it. This made it possible that the bone could be investigated in central direction together with the spongiosa, starting from the periosteal surface. Ten different bones were used for the measurement of hardness and altogether 250 measurements were performed on human and bovine bones.

* Authors wish to express their gratitude on this occasion to J. Bálint, senior research associate of the National Traumatological Institute, for his valuable assistance in the experiments.

Results

Corticalis

Measurements were started from the periosteal surface and it was experienced that the values of hardness were rather fluctuating near the surface in comparison with the central parts. The extreme values measured on laminae speciales were 50.2 and 26.3 in contrast to the average value of 37.4 characteristic of corticalis. Similar great oscillations were not observed on other parts of the corticalis. The restless sphere extends to about 0.4 to 0.6 mm from the periosteal surface. The values observed on the peripheral part are represented in a scatter diagram in Fig. 1.

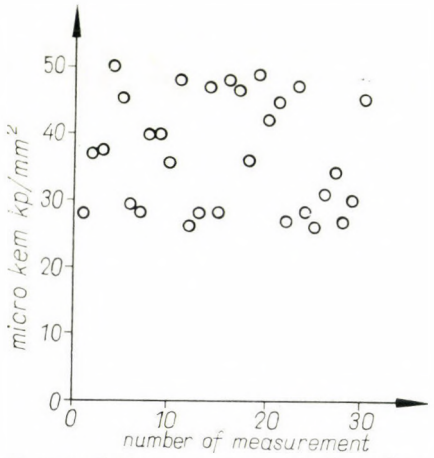

Fig. 1. Scatter diagram. Scatter of 30 measurements performed on the peripheric part of the corticalis (0.0 to 0.6 mm). Minimum value: 26.3, maximum value: 50.2

Intense decrease of hardness was experienced while investigating osteons located more centrally near the canalis centralis. In the immediate vicinity of the canalis the hardness decreased to such a measure that its value was not measurable. The laminae speciales farther from the canalis centralis, did not differ significantly from the average values (Figs 2 and 3).

By decreasing the loading, theoretically the size of the impressure can be also decreased so much that it falls between the two laminae speciales and thus the difference of hardness of the laminae speciales as well as that of the matter between them can be measured. In reality, the decreasing of loading on the diamond pyramid together with the decreasing of the size of the impressure would decrease its geometrical characteristic and precise measurability to such degree which would make impossible the indicated measurement.

The hardness of the intercalary laminae was variable. The results of measurements performed by Palma and co-workers were confirmed only with certain restraint by our own measurements. According to our observations it is true though that the hardest regions are observable in the laminae intercalares,

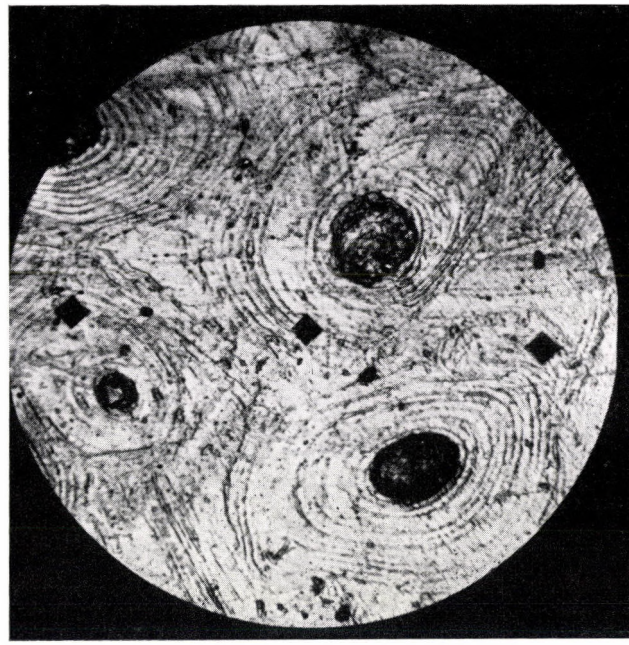

Fig. 2. Osteon system of human deep frozen bone conserve (tibia with three impressures of hardness measurements (×160))

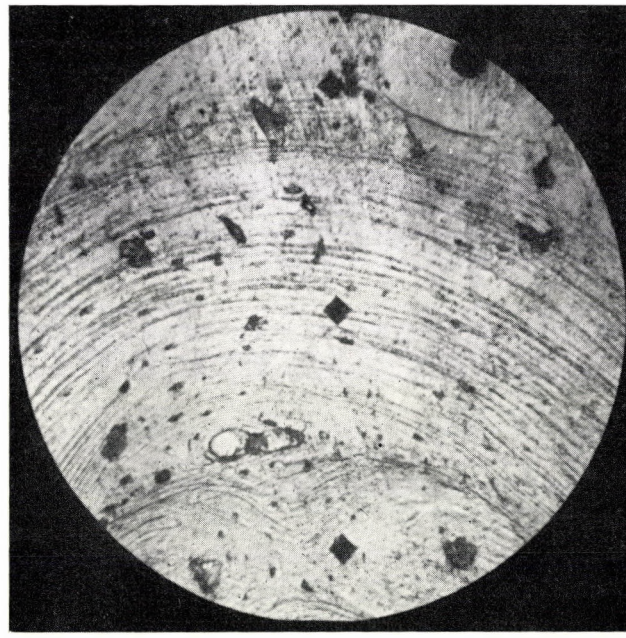

Fig. 3. Bovine bone conserve of Kiel with three impressures of hardness measurements (tibia corticalis) (×160)

nevertheless, this is not a general phenomenon. Measurements have shown that the average hardness of these laminae does not differ from the average hardness of the laminae speciales, however, intense values (54.6) were observed at some places. Consequently, the hardest parts of bones can be found in the region of laminae intercalares.

Table 1 shows the microhardness of a part of human conserved bone composed entirely of corticalis. The values are shown on a section of 3 mm extending from the periosteal surface to the medullary cavity. Measurements were performed in every 0.2 millimeter, regardless which part of the microstructure came under the measurements carried out in regular intervals. Column y indicates the distance of measurement from the periosteal surface, column d_1 is the registered value of diagonal, column d shows the real value of the diagonal, and HV is the microhardness expressed in kp/mm^2.

Table 1

Microhardness of No. H/2 human conserved bone composed entirely of corticalis
The section is extending from the periosteal surface to the medullary cavity and is 3 mm long

y	d	d_1	HV	Remarks
0.2	0.96	28.8	50.2	
0.4	—	—	—	Due to cavity not measurable
0.5	1.01	30.3	45.5	
0.8	1.08	32.4	39.7	
1.0	1.03	30.9	42.7	
1.2	1.08	32.4	39.7	
1.4	1.09	32.7	39.1	
1.6	1.06	31.8	41.4	
1.8	1.06	31.8	41.4	
2.0	1.12	33.6	36.9	
2.2	1.16	34.8	34.5	
2.4	1.04	31.2	43.9	
2.6	—	—	—	Due to cavity not measurable
2.8	1.03	30.9	42.7	
3.0	—	—	—	Due to cavity not measurable

Table 2 shows the distribution of the 150 measurements performed on different parts of the corticalis, and its extreme values.

Table 2

The distribution of the 150 measurements performed on different parts of the corticalis and its extreme values

Place of measurement	Smallest hardness	Greatest hardness	Number of measurement	Average value
Periosteal surface (0.4 to 0.6 mm)	26.3	50.2	30	37.2
Osteon system	27.4	43.9	89	33.6
Lamina intercalaris	33.4	54.6	31	38.2

Spongiosa

Altogether 100 microhardness measurements were performed on bones of cancellous character. The trabeculae of spongiosa proved to be always wide enough for the purpose of microhardness measurements. On that part of spongiosa which is nearer the corticalis, it is found that hardness corresponds to the value of hardness of the corticalis, in some cases. The values decrease after this. According to the measurements the average hardness of the spongy bone substance is

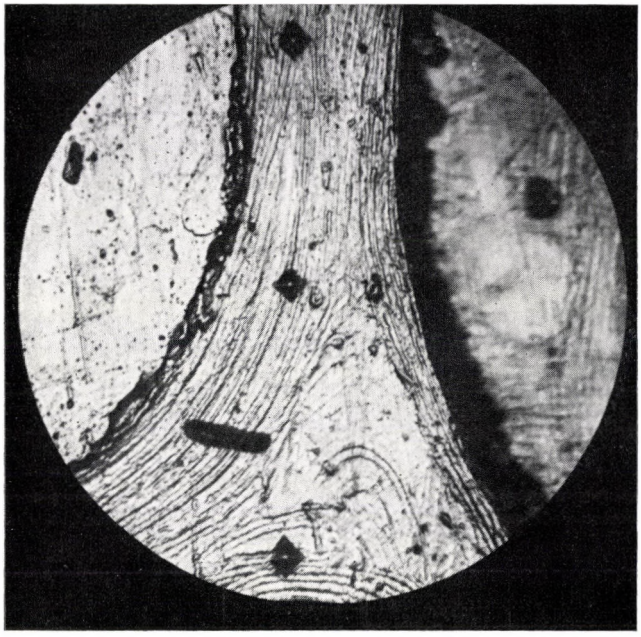

Fig. 4. Human deep frozen bone conserve (part of spongiosa) with three impressures of hardness measurements (× 160)

about 30 per cent smaller (26.3) than the average hardness of the corticalis. In the substance of spongiosa the sudden increase in the values is also observable (36.3) on some places similarly to the lamina intercalaris. However, these places are very definite and values about the average level are found again on the next place of measurement (Figs 4 and 5). The lowest measured value was 18.2.

Table 3 and 4 show the relations of microhardness of desantigenizated bovine conserved bone and of human deep frozen transplantate. The data refer to the 2 millimeter long cortical part of the bone extending from the periosteal surface towards the marrow cavity, as well as, the 2 millimeter long piece of spongiosa, extending from the boundary of corticalis spongiosa towards the marrow cavity. Measurements were performed at 0.2 millimeter distances along the linear axis of the bone regardless which part of the microstructure comes under the measurements carried out in regular intervals.

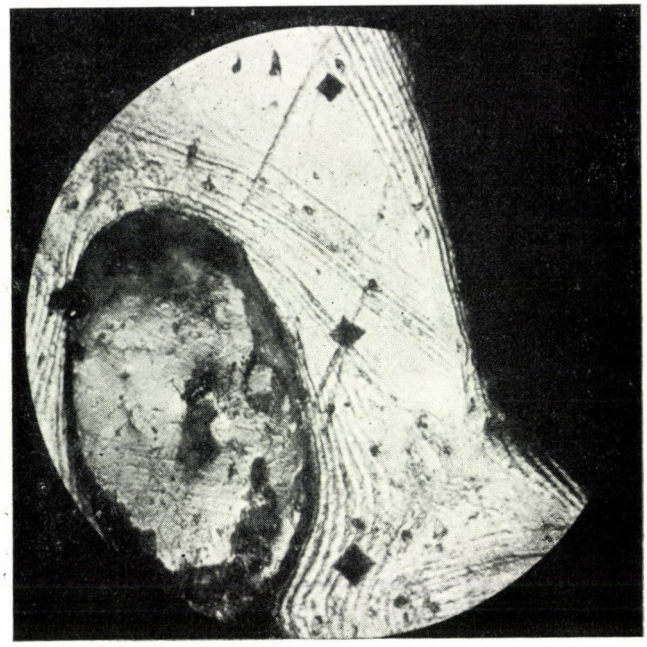

Fig. 5. Bovine bone conserve of Kiel (part of spongiosa) with three impressures of hardness measurements (×160)

Table 3

Microhardness of No. B/3 desantigenizated bovine bone conserves

y	d_1	d	HV	Remarks
0.2	1.01	30.3	45.5	
0.4	1.14	34.2	35.7	
0.6	1.22	36.6	31.1	
0.8	1.15	34.5	35.1	
1.0	1.11	33.3	37.6	
1.2	1.15	34.5	35.0	
1.4	1.17	35.1	33.8	
1.6	1.14	34.2	35.7	
1.8	1.18	35.4	33.4	
2.0	1.13	33.9	36.7	
				Boundary of corticalis-spongiosa
0.2	—	—	—	Due to cavity not measurable
0.4	1.30	39.0	27.4	
0.6	1.58	4.74	18.6	
0.8	—	—	—	Due to cavity not measurable
1.0	—	—	—	Due to cavity not measurable
1.2	1.29	38.7	27.9	
1.4	1.31	39.3	27.0	
1.6	—	—	—	Due to cavity not measurable
1.8	1.30	39.0	27.4	
2.0	1.75	52.5	15.2	

Column y shows the distance of measurements from the indicated boundaries, column d_1 indicates the registered value of the diagonal, column d is the real diagonal value and column HV shows the microhardness expressed in kp/mm^2.

Table 4

Microhardness relations of No. H/3 human deep frozen transplants

y	d_1	d	HV	Remarks
0.2	1.28	38.4	28.3	Periosteal surface
0.4	1.08	32.4	40.1	
0.6	1.08	32.4	40.1	
0.8	1.50	45.0	20.7	
1.0	1.12	33.6	36.6	
1.2	1.31	39.3	27.4	
1.4	1.11	33.3	37.8	
1.6	1.18	35.4	33.4	
1.8	1.07	32.1	40.7	
2.0	1.25	37.5	29.7	
				Boundary of corticalis-spongiosa
0.2	—	—	—	Due to cavity not measurable
0.4	1.24	37.2	30.2	
0.6	1.32	39.6	26.6	
0.8	—	—	—	Due to cavity not measurable
1.0	1.29	38.7	27.9	
1.2	—	—	—	Due to cavity not measurable
1.4	1.32	39.6	26.6	
1.6	—	—	—	Due to cavity not measurable
1.8	1.25	37.5	24.9	
2.0	1.23	36.9	30.7	

Human and bovine desantigenizated bones

In our earlier investigations the difference in hardness of these two types of bones was studied by the Brinell method. By this method only macroscopic measurements are possible. The bovine desantigenizated bone surpassed with about 5 per cent the human bone as regards Brinell-hardness. Measurements of microhardness do not indicate significant differences between the two bones notwithstanding that the desantigenizated bovine bone seems to be of different consistency to look at and to touch it, than the human conserved bone.

The analogy of mechanical properties of bone and copper

It is known that according to earlier experiments among metals mostly copper resembles the bone in stress analytical characteristics. This analogy can be confirmed on the basis of hardness measurements.

The value of hardness with soft copper is around 40 kp/mm^2. According to literary data and on the basis of our own measurements it can be stated that the average of Brinell and Vickers hardness, and microhardness values (37.4)

of the cortical bone are very near to the hardness of copper. The comparison applies, of course, only to the numerical data of the mechanical properties; resistance against active forces is ensured in an entirely different way, and with different structures by the two substances.

Discussion

Investigations on microhardness have shown that with the exception of the periosteal surface generally average values are found which are characteristic of the corticalis and spongiosa. The only exceptions are some well defined parts and the innermost part of the osteon.

According to literary data (Knese) the degree of calcification is higher in the inner lamellae of the osteon than in the outer ones. If hardness is regarded as the standard of mineralization then this statement is not verified by our investigations. Disregarding the part near the channel all the lamellae are of equal hardness with small differences.

The striking softness of the innermost part of the osteon deserves particular attention. This state can be explained most plausibly by the fact that due to the central channel the resistance of the surrounding substances is smaller against the active force. Nevertheless, it can be explained by the behaviour of the innermost lamellae differing from the others. It is known that the innermost lamella seems to be either generally or entirely homogeneous or the course of its fibre differs from the others. According to Knese here the collagen fibres are not entirely mature. Other authors demonstrated osteoid tissue in this place. The innermost lamella shows certain deviation in staining, too, and gives a characteristically indistinct picture. These differences obviously manifest themselves in the change of the ratio of the collagen – mineralized tissue which is manifested in the decrease of the values of hardness.

In some places the immensely great values of hardness of the lamina intercalaris can be related to the situation of these parts of bones and to the time of their development, as well as to the sequence of development. The intercalary laminae are comparatively farther from the place of remodelling. The appearance of the new and less mineralized tissues takes place in all probability to a slower measure in these regions. This shifts the proportions in favour of the mineralization together with an increase in local hardness.

On the occasion of transplantations it is a generally observed phenomenon that when remodelling has started the transplantation consisting of spongiosa remodels easier and faster than the transplanted corticalis. This difference is connected with the fact that much larger surface is taken by an identical volume in the case of spongiosa than of corticalis. On the basis of our experiments the question can be raised whether the smaller mineralization of the spongiosa does not contribute to an easier remodelling. The decision of this problem requires extensive and thorough experiments.

The fluctuations of hardness observed on the peripheric part of the bone support the view of Comtet and other authors, according to which the mechanical properties are not identical on the whole width of the corticalis. The objective phys-

ical investigation of this difference was not possible up to now because of the thinness of the layer and due to difficulties in the production of a specimen. Although hardness is only one of the physical properties of the substance, nevertheless, by the indication of the degree of mineralization it refers to the composition of the substance and thus to other physical characteristics as well. For example, the relation between the hardness of metals and tensile strength in technical mechanics is well known. In the case of bone, likewise, different values of hardness indicate changed composition and thus it means different physical properties.

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Effect of X-Irradiation on the Mitotic Cycle of NK/LY Ascites Cells Studied by Labelled Mitoses

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Authors used the labelled mitosis method to study on Németh—Kellner ascites lymphoma cells the radiosensitivity of the various phases of the mitotic cycle, following whole-body irradiation with 500 R of the host animal. The experiments were performed by the autoradiographic method, using H-3 thymidine. According to the results obtained, the generation cycle of NK/Ly ascites tumour cells changed after irradiation. They found phases G_2 and S to be prolonged on the day of irradiation and, owing to the G_2 block that developed, the cell population got partially synchronized. The postirradiation change of the parameters should always be taken into account when interpreting the results of experiments of a similar nature.

Introduction

Ever since the generation cycle of reproductive cells was subdivided into M, G_1, S and G_2 phases (Howard, Pelc, 1953; Lajtha, 1957), numerous papers have been published in this field on the radiosensitivity of these phases. According to investigations performed to date, any essential functional or eventual morphological change depends on the phase in which the cells were when irradiated. Accordingly, some workers set up the radiosensitivity sequence of the single phases as $S > G_1 > G_2$, others found phase G_1 to be more radiosensitive than phase S, others again found phase M to be the most radiosensitive. When studying L-cells, Dewey and Humphrey e.g. (1962) observed more chromosomal aberrations in cells irradiated in the S-phase than in cells irradiated in phases G_1 or G_2. In contradiction to that, three times more aberrations were found in Chinese hamster cells irradiated in phase G_2 (Hsu et al., 1962). Sinclair and Morton (1963) found synchronized Chinese hamster cells in phases M-G_1-G_2 to be rather radiosensitive, while cells in phase S less radiosensitive. Terasima and Tolmach (1963) set up the radiation sensitivity sequence of HeLa cells as M-S-G_2-G_1, while Brent, Butler and Crathorn (1966) found maximum radiosensitivity in phase S.

These contradictions might be explained mainly by the variety of the cell types studied. In addition, the different physical conditions (such as the dose delivered, dose rate, X-ray quality, etc.), the time elapsed between irradiation and the time of sampling, culture conditions (*in vivo* and *in vitro* experiments), the various testing methods applied, etc. might account for the differing results. The fact remains that the data obtained to date do not make it possible to set

up a generally valid radiosensitivity sequence, though all the authors are of the opinion that such a sequence really exists. The importance of the problem is only stressed by the metabolic processes known to proceed in the various phases. Accordingly, studies at a cellular level might help to approach the elucidation of radiation reactions, the revealing of the primary actions. The present study reports the postirradiation changes in the generation cycle of Németh–Kellner (NK/Ly) ascites cells as revealed by labelled mitosis.

Materials and Methods

NK/Ly ascites lymphoma cells were used as experimental objects (Németh, Kellner, 1960). The experiments were performed in the exponential growth phase of the ascites cells, on the 6th day following implantation. 3.10^7 tumour cells

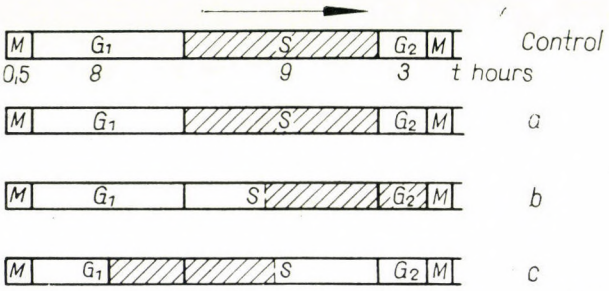

Fig. 1. Scheme of the cell cycle. Key: M = mitotic phase; G_1 = postmitotic rest phase; S = DNA synthesis phase; G_2 = premitotic rest phase. Within the cycle the cells pass from left to right. *Control.* Average time period in hours of the single phases in untreated NK/Ly ascites tumour cells. *a), b), c)* The hatched area represents the cells labelled with H-3 thymidine and their relative sites within the cell cycle when irradiated with 500 R

were injected intraperitoneally into each of 30 male white mice. On the 6th day following implantation, the mice were divided into 3 groups. Each group consisted of 6 irradiated and 4 nonirradiated mice. Physical conditions of irradiation: 500 R whole-body, 180 kV, 10 mA, 0.5 mm Cu-filter, FSD 60 cm, dose rate: 26.8 R/min. 10 μCi of H-3 thymidine (H-3 Tdr), dissolved in 0.2 ml of sterile physiological saline, were administered intraperitoneally to each mouse. (2.5 Ci/mM, TRA61, The Radiochemical Centre, Amersham, England.)

The animals in group 1 were given the DNA precursor immediately after irradiation. This administered DNA precursor was incorporated by the cells in the S-phase during irradiation (Fig. 1a). The animals in group 2 were irradiated at the time when part of the labelled cells reached the end of phase S and were in phase G_2 (Fig. 1b). A convincing evidence for this was furnished by the presence of active dividing cells in the smear prepared prior to irradiation (Fig. 4b). The irradiated animals of group 3 were whole-body irradiated with 500 R 4 hours before the administration of H-3 Tdr (Fig. 1c).

Thirty minutes after the administration of H-3 Tdr 0.1 ml of the ascites fluid was sucked off from the abdominal cavity of both the irradiated and the

nonirradiated animals. Further smears were prepared at 3 to 4 hours-intervals. The experiment lasted for 28 hours.

The smears were dried at room temperature and fixed for 40 min. in the mixture of acetic acid–methanol (1 : 3).

Autoradiography

The fixed smears were covered with a liquid emulsion (Ilford, Nuclear Research Emulsion, Type G 5), according to the method of Messier and Leblond (1957) and exposed to +4° C, for four days. Kodak's D 19 b developer was used for developing and an acid fixing bath for fixing. Rinsing with tap water for 30 minutes followed. Next, the wet autoradiograms were stained with 50 per cent May-Grünwald solution for 2 minutes and with 5 per cent Giemsa solution for another 5 minutes. 0.01 M phosphate buffer of pH 5.8 was used to dilute the staining solutions. After staining, the smears were rinsed with phosphate buffer and dried at room temperature. A few autoradiograms are shown in Fig. 2.

Evaluation

1000 cells were counted per smear to determine the labelling or thymidine-index. The ratio of active to inactive cells was expressed in per cents. When counting mitoses, the dividing forms between the early metaphase and the late anaphase were considered. The percentage distribution of labelled–nonlabelled dividing cells obtained at different intervals after irradiation is plotted in Figs 3 and 4. The characteristic curve obtained was evaluated according to the method recommended by Maurer and Koburg (1961), and by Wegener and Hollweg (1964), respectively. The time elapsed between thymidine administration and the appearance of the first labelled dividing cells represents phase G_2; the time interval between the beginning of the ascending slope of the curve and the end of the plateau represents phase S; the time period between the identical points of the curve represents the generation cycle.

The grain counts above the nuclei on smears prepared 30 minutes after the administration of H-3 Tdr were evaluated quantitatively, too.

Results

Fig. 3 shows the ratio of labelled to nonlabelled mitoses in the nonirradiated animals, as a function of time. No labelled dividing cells were found in the smears prepared 3.5 hours after the administration of H-3 Tdr. The first labelled mitoses appeared in about 4.5 hours and in smears prepared at later dates their number amounted to 85 to 95 per cent. The first ascending limb of the curve was extrapolated to the abscissa and 3.5 hours were obtained for phase G_2 + M. Upon further analysis of the curve 9 hours were obtained for phase S, 8 hours for G_1 and 20 to 21 hours for the generation cycle as a whole.

The mitotic index for nonirradiated NK/Ly ascites cells ranged from 1

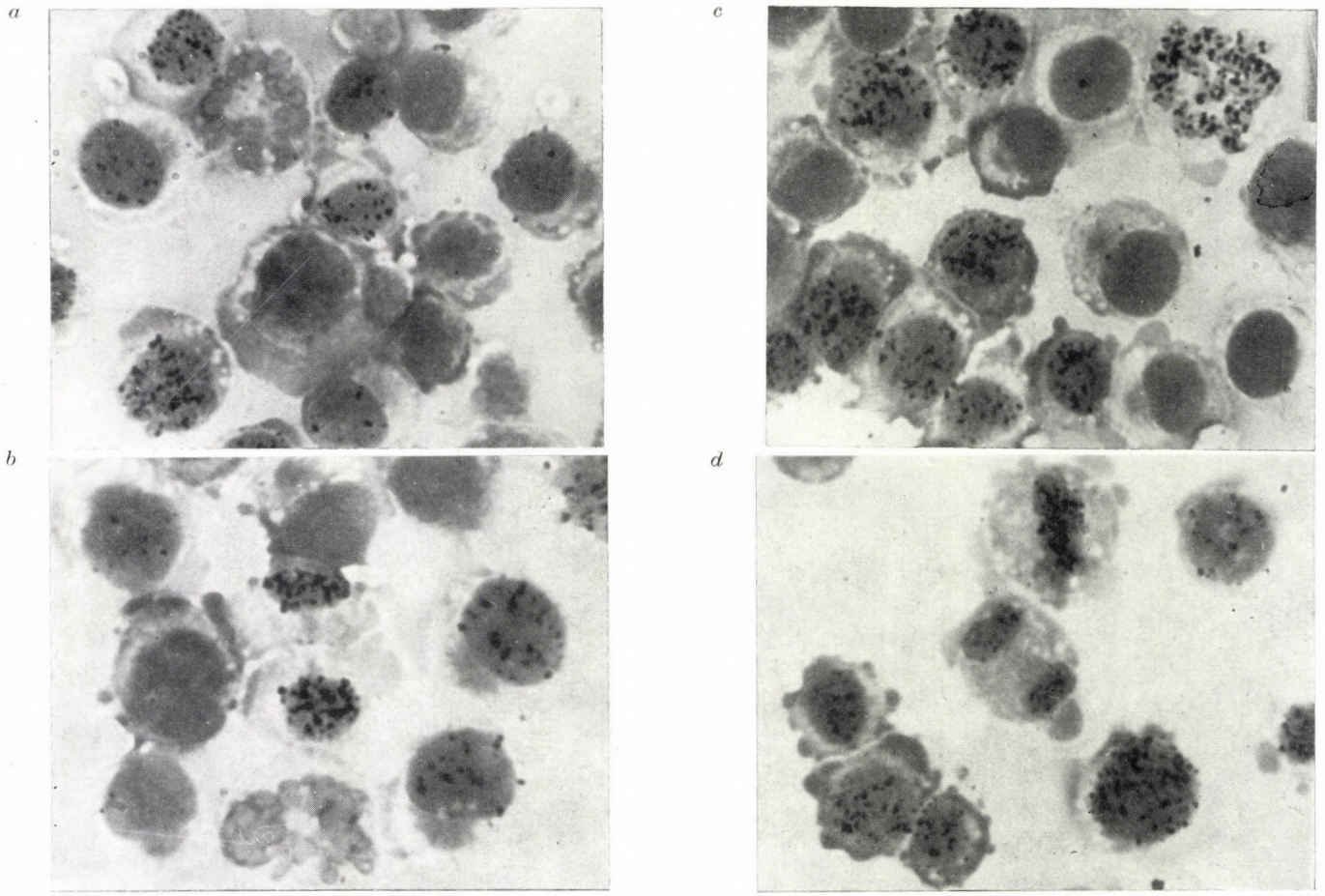

Fig. 2. Autoradiograms of smears obtained from the ascites fluid of the animals in group 1. Ilford, Nuclear Research Emulsion, Type G 5; May-Grünwald-Giemsa staining: *ab.* 1000×. *a, b:* control. *c, d:* irradiated. Smears were prepared 15 hours after H-3 thymidine administration

to 2 per cent and remained constant within these limits during the observation period.

In the X-irradiated animals of Group 1 (Fig. 4a), the first labelled mitoses appeared between the 9th and 10th hour. In the smears prepared 30 minutes after thymidine administration, the mitotic rate was not higher than 1 to 2 per mill and remained at this low level up to the 9th hour. Following the appearance

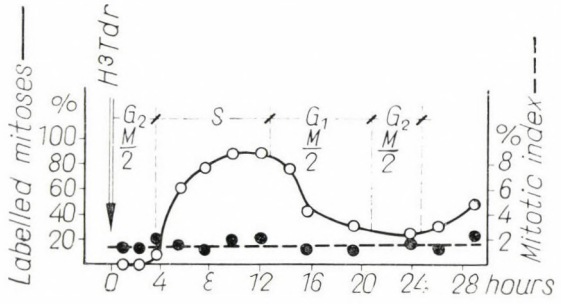

Fig. 3. Determination of the generation cycle and its phases by the labelled mitosis method in untreated NK/Ly ascites cells

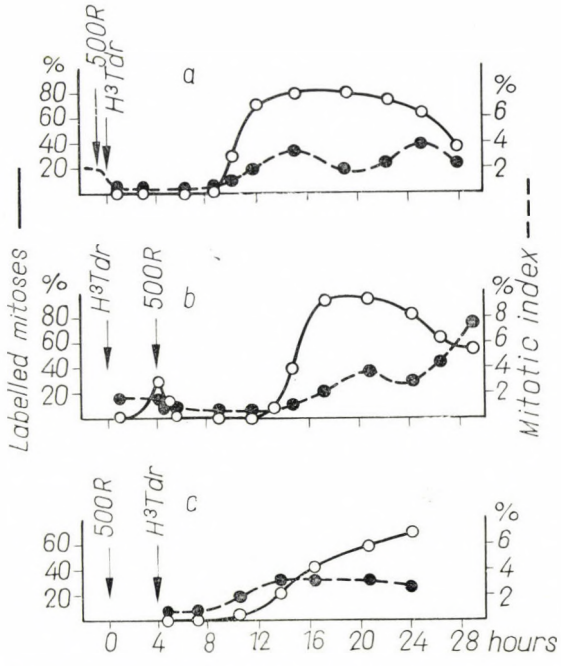

Fig. 4a—c. Percentage ratio of labelled and nonlabelled mitotic forms and the change of the mitotic index as a function of time in cells labelled with H-3 thymidine and irradiated with 500 R at different times

of the inactive mitotic forms, the number of labelled dividing cells also increased, reaching the maximum in the 11th to 12th hour. The mitotic rate increased to 3 to 4 per cent – as compared to the 1 to 2 per cent mitotic rate found in the controls.

The animals in Group 2 were X-irradiated when the labelled cells were in phase G_2 and phase M (Fig. 4b). In the smear prepared 3.5 hours after thymidine administration the ratio of labelled cells in mitosis was found to be 30 per cent. Under the effect of irradiation, the mitotic rate dropped to 1 to 2 per mill and no active divisions were found in smears prepared at later dates. Simultaneously with the rise of the mitotic rate, in the 13th to 14th hour of the experiment, labelled dividing cells also appeared. A marked increase of the mitotic index was observed also in this case.

The inhibition of mitosis, lasting also for 9 to 10 hours, could be observed also in the X-irradiated animals of Group 3 (Fig. 4c). In this group, the number of inactive dividing cells increased earlier, while the labelled mitotic forms appeared but 2 to 3 hours later. However, the slope of the curve is flat, it reaches hardly 70 per cent by the end of the experiment.

No appreciable differences were found in the thymidine- or labelling index values of the animals in groups 1 and 2, respectively. In the nonirradiated animals the thymidine index was found to be 40 to 45 per cent between 0 to 5 hours and increased to 45 to 55 per cent between 6 to 13 hours, when the active cells were in mitosis. This increase might be explained by the fact that, owing to their division, the active cells increased the cell count of the population in the period in question, while the count of inactive cells remained unchanged. This kind of increase went on as long as the inactive cells of phase G_1 passed into phase M.

A thymidine index of 40 to 45 per cent was found for the X-irradiated animals, too. The index remained constant during the entire period of mitotic inhibition. The rise of the thymidine index coincided with the rise of the mitotic rate.

A lower thymidine index of 35 to 40 per cent was found with the X-irradiated animals in group 3 and this value remained constant during the whole experimental period.

Similar differences appeared also in the grain counts over the nuclei in the smears of irradiated and nonirradiated animals in groups 1 and 2, respectively. The grain count displayed an average distribution, with frequency maxima of 25 to 30 grains/nucleus. On the other hand, values 5 to 10 grains lower were found for the X-irradiated animals in group 3. Accordingly, both DNA-synthesis rate was slower and the count of DNA-synthesizing cells was less in this group.

Discussion

In the experimental series reported in the present paper, the labelled mitosis method was used to follow the passage through phase M of NK/Ly ascites tumour cells labelled with H-3 Tdr in the DNA synthesis phase and X-irradiated with 500 R in the various phases of the generation cycle. All the

tests were performed on the very day of irradiation. Accordingly, we, indeed, studied the instantaneous effect of ionizing radiations in an *in vivo* system.

According to the results obtained, the immediate effect induced by X-irradiation manifested itself in mitosis inhibition, lasting for 9 to 10 hours in all three groups. After irradiation, the mitotic index dropped rapidly and 45 to 60 minutes afterwards the mitotic inhibition was completed. The pro- and metaphase-stage cells disappeared first from the cell population and the ana- and telophase stage cells the next. In smears prepared at later dates dividing cells occurred but sporadically. Concluding from the results obtained with mainly *in vitro* irradiated cultures, numerous authors (Puck, Steffen, 1963; Upton, 1963; Watanabe, Okada, 1966; Whitmore et al., 1961) interpret the induction of mitotic inhibition by a block developing at the end of phase G_2. Also according to our results, the mitotic process itself, at least that part of the M-phase which might be satisfactorily followed by light microscopy, does not seem to be radiosensitive. This is suggested by both the disappearance sequence of dividing forms and the time of development of the full inhibition. Cells sited behind the G_2 block enter the M-phase, while cells already in the M-phase continue their dividing processes and enter phase G_1. However, owing to the block of G_2, the replacement of dividing (M-phase) cells stops, and as soon as the average M-period is over, the cell population does not contain dividing forms any more.

However, the actual cause of the G_2 block has not been revealed so far: what may be the mechanism acting at a cellular level, the lag or inhibition of which arrests the mitotic process. Several authors (Upton, 1963; Whitmore et al., 1961) observed the inactivation of the centriolum under the effect of both physical and chemical agents (cold, hypoxia, cytostatica, etc.) and render also the effect of ionizing radiations probable by the failure to function of this cellular component.

The dividing forms appeared after the 9 to 10 hours mitotic inhibition and the mitotic rate displayed a slow rise. After 3 to 4 hours, counted from the restart of mitoses, the mitotic rate exceeded the control value. We interpret this maximum mitotic frequency by the fact that the distribution of the total cell count changes among the various phases of the cell cycle. Accordingly, they accumulate in phase G_2, before the section of G_2 block. Hence, as soon as the inhibition is released, more cells enter the M-phase and that is what increases the mitotic index. The changed distribution of cells, representing practically partial synchronization, manifests itself also in the change in the count of DNA-synthesizing cells, as indicated also by the thymidine index. The lower thymidine index obtained for group 3 (35 per cent) is also explained thereby. In this particular group, H-3 Tdr was administered only 4 hours after irradiation. The partial synchronization developed during these 4 hours accounts for the 20 to 22 per cent drop in the count of S-stage cells.

The thymidine indices found in groups 1 and 2, respectively, were identical with that of the control group. However, the partial synchronization developed in the animals of these two groups did not influence the thymidine index, since the animals were given X-irradiation and H-3 Tdr simultaneously (Group 1), or thymidine first and irradiation 4 hours afterwards (Group 2).

In smears prepared at later dates the thymidine index did not change appreciably. The slight fluctuation of the index was explained in detail in chapter Results. The steadiness of the thymidine index confirms that after the dose delivered, one hardly has to reckon with an immediate cell destruction. The change in the total cell number also developed correspondingly. In spite of the mitotic inhibition existing for 9 to 10 hours, the number of tumour cells did not decrease on the very day of irradiation, only from the 2nd day onward (Varga et al., 1966). Watanabe et Okada (1966) reported similar results after having irradiated *in vitro* cultured L cells with a dose of 1000 R and found appreciable cell death but after the second and third generation cycles.

The slopes of the labelled mitotic curves differed appreciably in the three irradiated groups (Figs 4a, b, c). The section representing the S-phase was prolonged in all three cases, however, to different extents. The shortest prolongation (10 hours) was obtained for group 2, a longer one (15 hours) for group 1 and the longest one for the irradiated animals of group 3. The ascending slope of the labelled mitotic curve for group 3 was so flat that the period of the S-phase could not be expressed numerically. Owing to the prolongation of the part-phases, the generation cycle itself also became longer.

The contradictory data in the literature on the study of reproductive cells might, at least in part, be explained by the changed mitotic cycle. Therefore, in experimental series of a like nature both the change of the mitotic cycle and the postirradiation changes of the cell counts in the various phases *should* always be taken into account and the results obtained should be evaluated correspondingly.

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Book Review

Механизмы интеграции клеточного обмена (Integrative Mechanisms in Cell Metabolism). Ed. S. A. Neifakh, Izd. Nauka, Leningrad, 1967, pp. 315.

The final purpose of biochemical studies, which make an effort to study the living processes in the simplest possible systems, is to draw conclusions regarding the complete system, that is the cell, the tissue or the organism. It is generally rather difficult to draw conclusions regarding something complicated from studies of simple, isolated systems. This is so because the individual factors do not simply add up, and new functions may appear. As pointed out by the editor and author of one of the chapters in the volume, S. A. Neifakh, such new functions are, among others, regulation and integration. Even the simplest processes may be certainly affected by these complex functions.

Actually, an explanation of the functional organization of the great variety of subcellular particles in which different regulatory mechanisms are operating is certainly a difficult task owing to the complex nature of the system. The monograph edited by S. A. Neifakh seems to be a successful attempt to give a survey of the functional properties of the different subcellular systems and complexes formed by the interaction of individual molecules.

The articles are dealing particularly with the transfer and coupling of energy (oxidative chain, glycolysis, photosynthesis, etc.), with localization of certain regulatory mechanisms, with the interactions among the different metabolic processes, and with the genetic control of the formation of structural elements. The investigations on the structure and function of membranes, carried out by the editor and his collaborators, should be specially noted.

The monograph contains six chapters: Chapter I (Dynamic, structural and genetic factors in the integration of metabolism, by S. A. Neifakh) introduces the problem on the basis of the recent advances in molecular biology. Chapter II (Structural and molecular organization of photosynthesis, by O. P. Osipova) deals with the structural and chemical organization of the process of photosynthesis. Chapter III (Structural organization of biological oxidation and coupled processes, by V. S. Gaitskhoki) is an up-to-date summary of the localization of interactions among the earliest known biochemical cycles and metabolic sequences. Chapter IV (The genetic control over synthesis, of structural elements of respiratory chain, by T. B. Kazakova) is discussing the genetic control of the regulatory mechanisms of metabolic processes. Chapter V (Coordination of respiration and glycolysis, by V. S. Repin) describes the molecular mechanism of the control of the functioning of individual enzymes and of multi-enzyme systems. Chapter VI (The energy coupling between oxidation and active transport, by N. K. Monakhov) deals with the role of biological membranes in the chemical processes of the organism, and also with the role of membrane in the formation of the steric structures of the cells, and presents a discussion of the steric orientation of chemical reactions.

The titles of the chapters show the high level at which the problems of biochemical integration are analyzed in this volume. This very up-to-date book which contains about 950 references, 68 figures and 20 tables is very useful for those who want information on the molecular mechanism of the chemical processes in the living cell.

P. ELŐDI

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Temporal Changes in the Distribution of Labeled Palmitic Acid in the Different Lipids of Rat's Adipose Tissue, Liver and Diaphragm

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Epididymal fat pads, diaphragm and liver slices of rats were incubated for 10 min in a medium containing 1-^{14}C palmitic acid. One group of tissues was then killed, while the other group was further incubated in inactive medium. Similar experiments were carried out also with in vivo incubation of epididymal fat pads. The distribution of radioactivity among the lipid classes was compared in the two groups.

In all tissues the bulk of fatty acids was incorporated very quickly, already while in the active medium, into the triglycerides. A considerable part of fatty acids went into the diglycerides. In the adipose tissue the activity of diglycerides fell both in vivo and in vitro only after about 1 hour to its half. In the diaphragm and liver slices reincubated in inactive medium the activity of diglycerides fell after 2 hours to its half.

It is supposed that there are two different pathways for the incorporation of fatty acids into triglycerides.

In the adipose tissue 38 per cent, in the diaphragm 39 per cent of the activity of triglycerides was found in the fully saturated triglycerides.

A method is described for the calculation of the mixing ratio of endogenous and exogenous fatty acids from the distribution of radioactivity between saturated and unsaturated triglycerides, making possible the determination of the rate of triglyceride synthesis.

Introduction

According to Weiss and Kennedy (1956) the pathway of triglyceride synthesis is:

fatty acid + ATP + CoA → fatty acyl − CoA + AMP + PP

2 fatty acyl − CoA + α-glycerophosphate → phosphatidic acid →

→ diglyceride + P

diglyceride + fatty acyl − CoA → triglyceride + CoA

Data of several papers refer to the fact that, when labeled fatty acid was given to homogenates of different tissues or to in vitro incubated tissue slices, significant activity could be observed in diglycerides. Changes in diglyceride activity as a function of time were not investigated and in general the presence of radioactive diglycerides was accepted on the basis of the above scheme.

In an earlier work (Herodek, 1967) I reported that by incubating the adipose tissue with labeled palmitic acid the ratio of the radioactivities of diglycerides and

triglycerides did not change significantly during incubation. The activity of triglycerides was about twice as much as that of diglycerides. Even if the tissue, incubated first with labeled palmitic acid, was reincubated in an inactive medium for further 10 min, there was only a very small and not significant decrease in the radioactivity of diglycerides. These results indicated that the diglycerides are not to be regarded simply as precursors of a very rapid triglyceride synthesis, and that further investigations of their metabolism are necessary.

In the present work the half life time of diglycerides was determined in the in vitro incubated adipose tissue by increasing the incubation time in the inactive medium. Similar experiments were carried out with Steins' "in vivo" incubation technique in order to observe also under in vivo conditions the behaviour of diglycerides. Furthermore the changes in the activity of lipid fractions of diaphragm and liver slices incubated first in vitro with labeled fatty acid then reincubated in inactive medium were studied to decide whether the phenomenon observed in the adipose tissue is a specific property of this tissue alone or else is a general characteristic of fatty acid incorporation.

Materials and Methods

In the experiments male Wistar rats of 200 g were used. The animals were fed ad libitum on a standard diet. For experiments where in vitro incubated tissues were used the animals were killed by a blow on their head then their liver, diaphragm and epididymal fat pads were excised. Thin liver slices were prepared with a razor blade. The diaphragm was thoroughly cleaned from other tissue elements and cut sagittally into 2 symmetric halves. Of the epididymal fat pads the distal pieces weighing 150–200 mg were used. With adipose tissue and diaphragm the tissue pieces from the other side of the same animal, in the case of the liver, slices prepared from the same liver served as controls. First all tissues were incubated in a medium containing labeled palmitic acid for 10 min. Thereafter the tissues were removed from the radioactive medium with pincers, quickly rinsed with physiological saline, placed in an inactive medium and further incubated. One group of the adipose tissue was incubated for 5 min, the other for 1 hour in the inactive medium. In case of liver and diaphragm the first group was incubated for 10 min, the second group for 2 hours in the inactive medium. The tissues were incubated at 37°C by gentle shaking, the adipose tissue under air, the diaphragm and liver slices under O_2 atmosphere. Adipose tissue and diaphragm were incubated in 3 ml, the liver slices in 5 ml medium. The inactive medium consisted of 5 per cent bovine albumin and 0.09 per cent glucose in Krebs-Ringer-phosphate buffer (Ca^{2+} omitted), pH 7.4. The active medium contained in addition 2 μmoles/ml labeled fatty acid bound to albumin. 1-^{14}C palmitic acid (Reanal, Budapest) was used in the experiments with a specific activity of 1 mCi/mmole, consequently the specific activity of the active medium was 2 μCi/ml. The labeled fatty acids were bound to albumin by saponifying first the fatty acid with a small

excess of 0.1 N NaOH. This soap solution of a very small volume was warmed until clear then the phosphate buffer containing albumin was heated at 37°C and poured quickly into the soap solution. The mixture was vigorously shaken and filtered.

In some experiments the epididymal fat pads were incubated in vivo. In these cases the method of Stein and Stein (1962) was used.

After incubation the tissues were again rinsed with physiological saline, quickly weighed and homogenized in 25 ml of a chloroform-methanol mixture (2 : 1) in a Potter Elvehjem all glass tissue grinder. The extraction of lipids was performed according to Folch et al. (1957). An aliquot of the extract was evaporated and the radioactivity was measured to determine the total activity taken up by the different tissues.

Another part of the lipids was resolved by thin layer chromatography into their components in order to determine the percentage distribution of the radioactivity in the different lipid classes. Glass plates (20 × 20 cm) were covered with a silica gel (Kieselgel G nach Stahl, Merck) layer, 0.3 mm thick. A maximum of 30 mg lipid was applied to each plate in the form of a streak. The lipids were dissolved in 0.3 ml benzene and dripped with a micropipette on the plates. To locate the free fatty acids and diglycerides on the chromatogram 0.1 per cent inactive palmitic acid and 0.2 per cent inactive diglyceride were also added to the benzene in which the lipids were dissolved. The developing solvent consisted of petroleum-ether (B.p. 40–70° C) -diethyl ether-acetic acid (70 : 30 : 1). Under these conditions the following lipid fractions were separated on the chromatoplate: cholesterol esters, triglycerides, free fatty acids, diglycerides and monoglycerides + phospholipids. Monoglycerides and phospholipids remained at the start line. From this zone the silica gel was scraped off with a razor blade and the lipids were extracted from it first with 20 ml of a mixture of chloroform-methanol-water (12 : 6 : 1) by shaking for 10 min, then the solvent was decanted and the procedure repeated with another 12.5 ml volume of the same mixture. A third extraction was performed with 12.5 ml methanol-ammoniumhydroxyde (10 : 1) in the same way, thereafter the silica gel was rinsed with 12.5 ml methanol-ammoniumhydroxide. The solvents were pooled and evaporated. The other parts of the chromatograms were sprayed with ethanol containing 0.2 per cent Rhodamine-B. The bands corresponding to the different lipid classes were located under UV light and the silica gel from each band was scraped off separately. In the case of these bands the lipids were extracted from the silica gel with diethyl-ether in a small, 25 ml Soxhlet apparatus for two hours.

In certain cases the triglycerides from one part of the lipids were prepared by using the above described method and then the different triglycerides were separated according to the number of double bonds per molecule by means of silver nitrate thin layer chromatography (Barrett et al., 1963). As developing solvent benzene was used. In this case, too, the chromatogram was sprayed with Rhodamine-B and the different bands were extracted with diethyl-ether as described for normal silica gel plates.

Radioactivity measurements were carried out with a Packard Tri-Carb scintillation spectrometer. The lipid samples were dissolved in 10 ml scintillation solution consisting of toluene, containing 0.4 per cent 2,5-diphenyloxazole and 0.01 per cent 1,4-di-[2-(5-phenyloxazolyl)]-benzene.

Results

Table 1 shows the results of an experiment in which both epididymal adipose tissues of animals were first incubated for 10 min with 1-^{14}C palmitic acid then the adipose tissue from the one side was incubated in inactive medium for 5 min and that from the other side for 1 hour (Table 1).

Table 1

Changes in the percentage distribution of 1-^{14}C palmitic acid in adipose tissue during 1 hour incubation in inactive medium

Values are the means of 3 animals ± S.E. of the mean

	Time after labeling	
	5 min	1 hour 5 min
Triglycerides	77.6 ± 0.8	90.3 ± 0.6
Free fatty acids	1.9 ± 0.4	1.0 ± 0.2
Diglycerides	20.1 ± 0.4	8.4 ± 0.3
Other lipids	0.4 ± 0.0	0.3 ± 0.0

By incubating the control group in inactive medium for a few minutes it was achieved in all experiments that those free fatty acids which are always present in a considerable amount after the removal of the tissue from the media containing the labeled fatty acid, have already disappeared and thus the further incorporation from the free fatty acids could not change the ratio of activity of diglycerides and triglycerides. The results show that during incubation for 1 hour in inactive medium the radioactivity of diglycerides fell to about the half.

This experiment shows the fate of labeled diglycerides in the adipose tissue in vitro. The question as to the fate of labeled diglycerides under in vivo conditions still remained open. In the case of intravenous injection of the labeled fatty acid only a very small proportion of it appeared in the adipose tissue. For this reason Steins' (1962) in vivo incubation technique was chosen. With this method only the adipose tissue gets labeled and the possibility of transfer of labeled fatty acids from other tissues into the adipose tissue is excluded. The abdominal cavity of the anaesthetized animals was opened at the level of the outer inguinal ring and the two epididymal fat pads were suspended for 10 min in the same incubation solution as used in the in vitro experiments. Thereupon the adipose tissues were

washed with physiological solution and replaced into the animals. The incisions were sutured.

In Table 2 results of an experiment are given where the adipose tissue from one side was homogenized 5 min and that from the other side 1 hour after the incubation. This experiment was exactly the in vivo parallel of the in vitro experi-

Table 2

Changes in the percentage distribution of 1-^{14}C palmitic acid in adipose tissue in vivo during 1 hour

Values are the means of 4 animals ± S.E. of the mean

	Time after labeling	
	5 min	1 hour 5 min
Triglycerides	70.8 ± 2.0	83.6 ± 2.2
Free fatty acids	0.8 ± 0.2	0.6 ± 0.2
Diglycerides	28.2 ± 1.9	15.7 ± 2.2
Other lipids	0.2 ± 0.1	0.1 ± 0.0

ment demonstrated in Table 1 with the difference, that instead of the common in vitro incubation the labeled palmitic acid was administered with Steins' technique into the adipose tissue and that in lieu of an incubation in inactive medium the adipose tissue was replaced to its anatomical place, maintaining its normal innervation and blood supply. It is shown that the two different experiments gave very similar results. In vivo, too, about 1 hour is needed that the radioactivity of diglycerides be diminished to its half.

In Table 2 the activity of diglycerides in the control group is higher than in Table 1. However, this does not mean differences between the in vivo and in vitro results as in other in vitro experiments (Herodek, 1967) a 28 per cent activity was observed in diglycerides and also in vivo activities below 20 per cent were found in diglycerides e.g. in the experiment given in Table 3. The results published

Table 3

Changes in the percentage distribution of 1-^{14}C palmitic acid in adipose tissue in vivo during 5 hours

Values are the means of 4 animals ± S.E. of the mean

	Time after labeling	
	5 min	5 hours
Triglycerides	80.0 ± 0.6	96.7 ± 0.2
Free fatty acids	1.2 ± 0.1	1.0 ± 0.2
Diglycerides	18.5 ± 0.4	2.1 ± 0.3
Other lipids	0.3 ± 0.1	0.2 ± 0.0

here but also those of other experiments show that the distribution of the label in animals used in the same experiments is very similar while the averages of experiments carried out at different times may differ, the activity of diglycerides varying between 16 and 30 per cent in different experiments. The cause of these variations is not yet clear.

In the following experiment (Table 3) the same method was used as before with the exception that the adipose tissue from the one side was removed from the animals after 5 hours instead of 1 hour following incubation. A further decrease in the activity of diglycerides is visible.

In Table 4 results of an experiment are given where one fat pad was removed 1 day, the other 6 days after the incubation. It can be seen that even after 1 day

Table 4

The percentage distribution of 1-^{14}C palmitic acid in adipose tissue 1 and 6 days after the "in vivo" incubation

Values are the means of 4 animals ± S.E. of the mean

	Time after labeling	
	1 day	6 days
Triglycerides	97.3 ± 0.1	99.0 ± 0.1
Free fatty acids	1.7 ± 0.2	0.5 ± 0.1
Diglycerides	0.8 ± 0.0	0.4 ± 0.1
Other lipids	0.2 ± 0.0	0.1 ± 0.0

the activity of diglycerides did decrease and on the 6th day 99 per cent of the total activity was already in the triglycerides.

In short it was found that in the case of adipose tissue the bulk of labeled fatty acids both in vitro and in vivo was incorporated within a very short time into the triglycerides while their other, considerable part went into the diglycerides, which at the end, were also transformed into triglycerides, this latter being a rather slow process. It still remained an open question whether this duality of the incorporation of fatty acids into triglycerides is characteristic only of the adipose tissue or it is a common feature of triglyceride synthesis in general.

In order to clear the problem the investigation was extended to muscle and liver tissues, too. According to some preliminary experiments the life time of diglycerides is in liver and muscle tissues longer than in the adipose tissue; for this reason a postincubation for 2 hours was performed.

In the case of both tissues the control group was kept for 10 min in inactive medium following the 10 min long incubation in the labeled medium in order to give time for the labeled free fatty acids to get incorporated into the esters. The other group of tissues was incubated in inactive medium for a further 2 hours. Data for the diaphragm are presented in Table 5, those for the liver in Table 6. In these tissues in contrast to the adipose tissue there is a considerable activity

also in the fractions of cholesterol esters and phospholipids + monoglycerides. During the 2 hours in inactive medium the radioactivity of diglycerides fell in both tissues to the half.

During the 10 min incubation the fatty acid uptake of the different tissues from the medium containing labeled fatty acids calculated for 1 gm fresh weight was the following: adipose tissue 0.736 ± 0.015 μmole, diaphragm: 0.094 ± 0.008

Table 5

Changes in the percentage distribution of 1-^{14}C palmitic acid in the diaphragm during 2 hours incubation in inactive medium

Values are the means of 3 animals ± S.E. of the mean

	Time after labeling	
	10 min	2 hours 10 min
Cholesterol esters	1.5 ± 0.1	1.0 ± 0.1
Triglycerides	58.7 ± 2.3	74.8 ± 1.4
Free fatty acids	2.6 ± 0.7	1.2 ± 0.1
Diglycerides	25.2 ± 1.1	12.6 ± 1.4
Phospholipids + monoglycerides	12.0 ± 0.8	10.4 ± 1.9

Table 6

Changes in the percentage distribution of 1-^{14}C palmitic acid in liver slices during 2 hours incubation in inactive medium

Values are the means of 3 animals ± S.E. of the mean

	Time after labeling	
	10 min	2 hours 10 min
Cholesterol esters	6.3 ± 0.9	3.9 ± 0.3
Triglycerides	61.2 ± 2.2	68.0 ± 2.7
Free fatty acids	2.1 ± 0.1	1.2 ± 0.2
Diglycerides	14.2 ± 1.8	6.8 ± 0.4
Phospholipids + monoglycerides	16.2 ± 1.5	20.1 ± 3.1

μmole, liver: 0.323 ± 0.037 μmole. In the course of postincubations lasting for 1 and 2 hours, respectively, no significant decrease of radioactivity was found in these tissues.

Adipose tissues from 3 animals were incubated in inactive medium for 10 min then they were immediately homogenized, without any postincubation. In this case during the 10 min in the radioactive medium 0.51 ± 0.02 μmole labeled fatty acid/gm living tissue was incorporated into triglycerides.

The triglycerides were prepared from the lipids of adipose tissues incubated in active medium for 10 min then for 5 min in inactive medium (identical with the control group in Table 1) and from the lipids of the diaphragms incubated for 10 min in active then for 10 min in inactive medium (identical with the control

Table 7

The percentage distribution of 1-^{14}C palmitic acid in triglycerides separated according to the number of double bonds per molecule

Values are the means of 3 animals ± S.E. of the mean

Number of double bonds in the triglyceride molecule	Adipose tissue	Diaphragm
0	37.9 ± 0.1	39.2 ± 0.6
1	35.7 ± 0.7	36.7 ± 0.5
2	16.9 ± 0.3	16.7 ± 0.5
>2	9.5 ± 1.0	7.4 ± 0.6

group in Table 5). These triglycerides were chromatographed on silver nitrate impregnated chromatoplates. In this way the distribution of radioactivity in the triglycerides separated according to the total number of double bonds per molecule was determined (see Table 7). In both tissues a very high activity was found in the fully saturated triglycerides. The interpretation of this phenomenon will be given in the discussion.

Discussion

By incubating adipose tissue, diaphragm and liver slices in vivo or adipose tissue in vitro in media containing labeled fatty acids it was equally found that the major part of the labeled fatty acids taken up by the tissue was already incorporated into the triglycerides at the end of the 10 min incubation period. In the different tissues the radioactivity of diglycerides accounted for about 20 per cent of the total activity and this activity fell to its half in the case of adipose tissue in 1 hour and in cases of diaphragm and liver slices in 2 hours. In a first attempt to reconcile these findings with the Weiss–Kennedy pathway of triglyceride synthesis one could suppose that the bulk of fatty acids entering the adipose tissue does not combine mainly with the newly formed diglycerides to form triglycerides but are attached to the diglycerides preexisting in the tissue. If the diglyceride pool is large enough it ensures in spite of the long life time the appropriate turn over rate for the triglyceride synthesis. According to this assumption the labeled fatty acids should occur in the triglycerides mostly combined with fatty acids of the tissues. The results, however, presented in this paper show just the opposite. In the

adipose tissues used in these experiments exactly 33 per cent of the fatty acids were saturated. If from this one-third of saturated fatty acids and from the two thirds of unsaturated fatty acids the diglycerides were formed randomly in the tissue, then only 10 per cent of the diglycerides would include both fatty acids in a saturated form. According to enzymological investigations the enzymes involved in the formation of phosphatidic acid do not distinguish between saturated and unsaturated fatty acids (Lands, Hart, 1965) and it was found in experiments with tissue slices (Herodek, 1967) that the fatty acids are randomly distributed in the triglycerides. With regard to these observations it seems rather improbable that the amount of diglycerides containing two saturated fatty acids could exceed considerably 10 per cent of the total diglycerides. On the other hand it is obvious that a fully saturated triglyceride can be formed only from those diglycerides whose both fatty acids are saturated. However, as shown above the fully saturated triglycerides contained 38 and 39 per cent of the total activity of triglycerides in the adipose tissue and diaphragm, respectively. It would be difficult to give another interpretation as that the palmitic acid molecule taken up by the tissues participate in a rapid de novo triglyceride synthesis whilst only a limited dilution by the tissue fatty acids takes place and that they build up tripalmitate mainly by combining with each other.

Another possibility, notably that the newly synthesized triglyceride would quickly degrade to diglyceride, then the latter would become again triglyceride and so on, – i.e. that the lasting label in diglycerides would be sustained by a dynamic equilibrium between triglycerides and diglycerides could be excluded by earlier experiments (Herodek, 1967).

If the rapidly labeled triglycerides were produced via the Weiss–Kennedy pathway by de novo synthesis, then they would have to be formed through very short-lived diglycerides. Therefore, it seems that two different diglycerides are formed in the tissues in the course of fatty acid uptake, – one that turns immediately into triglyceride and another, from which triglyceride is formed only after some hours.

Lands and Hart (1966) suggest also another way of triglyceride synthesis involving the formation of lecithin. In this way diglyceride can be formed from both phosphatidic acid and lecithin. It seems possible that the diglycerides of the two kinds of origin do not mix – but one is converted into triglyceride and it is the other one whose lasting radioactivity was observed in these experiments.

As it has been reported earlier (Vaughan et al., 1964) up to now it was impossible to determine the intensity of triglyceride synthesis in the adipose tissue from the incorporation of the labeled fatty acids as the mixing ratio of endogenous and exogenous fatty acids, i.e. the degree of the dilution of the labeled fatty acids in the adipose tissue by its own, unlabeled fatty acids – and therefore the specific activity of the fatty acid pool was not known. In turn a nonisotopic method was introduced (Vaughan, 1962) to measure the rate of reesterification of fatty acids. The adipose tissue releases less fatty acid than should be equivalent to the glycerol release, the difference being reesterified by the adipose tissue.

The intensity of reesterification was found to be 3.8 μmoles/g living tissue/hour. These calculations were based on the hypothesis that the once liberated glycerol cannot be reutilized by the adipose tissue because it does not contain glycerokinase. Recently it has been stated (Robinson, Newsholme, 1967) that glycerokinase is present in the adipose tissue, therefore the calculation based on the glycerol–fatty acid equilibrium underestimates to a certain degree the intensity of the reesterification of fatty acids.

Supposing that in the process of triglyceride synthesis the different fatty acids are distributed randomly in the molecules a simple relation between the ratio of saturated to unsaturated fatty acids in the triglyceride synthesis pool and the distribution of the labeled fatty acid in saturated and unsaturated triglycerides can be deduced (Herodek, 1967). If T represents the radioactivity of trisaturated triglycerides in per cent of the radioactivity of the total triglycerides and s stands for the quantity of saturated fatty acids in per cent of total fatty acids, the relation can be formulated as: $T = \frac{s^2}{100}$. An experiment where s was known, resulted in a T value corresponding to the above formula and supporting the validity of the calculation. Now the procedure will be reversed; from the radioactivity of saturated triglycerides the ratio of saturated and unsaturated fatty acids in the synthesis pool is calculated. The radioactivity of triglycerides containing 3 saturated fatty acids amounts to 39 per cent of that of total triglycerides. Substituting this value into the $s = 10\sqrt{T}$ formula; $s = 10\sqrt{38} = 62$, i.e. in the triglyceride synthesis pool 62 per cent of fatty acids is saturated. From this value again the mixing ratio of fatty acids taken up from the medium and those of the tissue, that is the value missing for the calculation of the intensity of the triglyceride synthesis from the incorporation of labeled fatty acids can be calculated. All 38 per cent of the unsaturated fatty acids originated from the adipose tissue, as in the medium only palmitic acid, i.e. saturated acid was present. The 38 per cent unsaturated fatty acids of tissue origin are paralleled by 19 per cent saturated fatty acids of tissue origin because within the adipose tissue one third of fatty acids is saturated. Thus 43 per cent remains for fatty acids taken up from the medium. In these experiments 0.51 μmole fatty acid was incorporated into triglycerides by the adipose tissue per g within 10 min. Converted for 1 hour, this means 3.1 μmoles fatty acid/g living tissue/hour. Consequently the intensity of the total triglyceride synthesis in the adipose tissue is 7.1 μmoles fatty acid/g living tissue/hour. From this quantity 4 μmoles fatty acid/g living tissue/hour is due to the reesterification of the tissues' own fatty acids.

This result is in very good agreement with the value calculated on the basis of the glycerol–fatty acid balance, indicating that in the intact adipose tissue the glycerokinase enzyme cannot be very active.

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Studies on Protein Complexes of Muscle by Means of Proteolysis

IV. Further Data on the Tryptic Digestion of Myosin in the Presence of Ca^{2+} Ions

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The authors studied the proteolytic fragments formed from myosin when digested at low ionic strength in the presence of 0.01 M $CaCl_2$. The protein fraction which is dissolved in the course of digestion is composed of HMM** and of HMM subfragment-1 (by gelfiltration and other criteria). The undissolved residue gives two major hypersharp peaks in the ultracentrifuge. The first peak is indistinguishable from myosin. The second peak closely follows the myosin peak (S^0_{20} cca. 5.6—5.8) and binds to actin like myosin. It is clearly a hitherto unknown tryptic derivative of myosin.

Introduction

There is a renewed interest in the fragmentation of myosin and of the meromyosins by proteolysis and also by some other treatments (Mueller, 1965; Young et al., 1964, 1965; Kominz et al., 1965; Lowey et al., 1967; Tokuyama et al., 1966; Lowey, 1964; Dreizen et al., 1967, etc.). We do feel, therefore, that it is worth giving an account of our recent experiments concerning the tryptic digestion of myosin, which we have carried out in the presence of Ca^{2+} ions at low ionic strength.

Lowey et al. (1967) fragmented myosin in a heterogeneous system consisting of an insoluble trypsin derivative and dissolved myosin. We have used aggregated myosin and dissolved trypsin and in this system we have also found that the composition of the digest is rather complex. In addition to intact myosin and the "traditional" fragments of myosin (HMM, HMM-subfragment-1 and LMM), there is an important component which cannot be identified with any of the hitherto known myosin fragments.

Methods

Myosin was prepared according to Portzehl et al. (1950), actin according to Bárány et al. (1954) and Mommaerts (1952).

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** List of abbreviations: LMM = light meromyosin; HMM = heavy meromyosin; DFP = diisopropyl fluorophosphate.

The trypsin used was a lyophilized Hungarian preparation (Reanal), which had been incubated in 0.01 M HCl for 16 hours at 37°C to destroy the chymotrypsin contamination. The trypsin : myosin ratio was 1 : 125.

Digestion was carried out in the presence of 0.04 M borate buffer, pH 8.8, and 0.02 M KCl plus 0.01 M $CaCl_2$. The treatment generally lasted for 20 minutes and it should be noted that the pH was slightly higher than the one in our previous paper. Myosin was first dialyzed against the same borate buffer supplemented with 0.02 M KCl. $CaCl_2$ was added to the suspension just prior to trypsin. We applied DFP to stop trypsin action in a final concentration of 0.01 M. The stock solution of DFP was prepared with a minimal volume of isopropanol and neutralized with alcoholic KOH. DFP was removed from the digest by dialysis for 24 hours against the $KCl-CaCl_2$-borate saline, used for digestion. Dialysis was followed by centrifugation for 30 min with 10 000 *g*. Ca^{2+} was removed from the supernatant by dialysis, and from the precipitate by adding iminodiacetate resin in the potassium phase (after having dissolved it in 0.5 M KCl), (see Biró et al., 1966). The precipitate fractions were re-precipitated several times by dialysis against 0.02 M KCl, 0.02 M borate (pH 8.2) followed by addition of 0.01 M $CaCl_2$.

Gel-filtration was carried out at 0–5°C with Sephadex G-200 (Pharmacia) on a 110×1.4 cm column, which was equilibrated with 0.5 M KCl and 0.1 M tris-HCl, pH 7.5. The rate of flow was about 8 ml/h. The column was charged with about 30 mg protein in 4 ml solution.

For thin-layer gel-filtration we used the "fine" fraction from Sephadex G-200 passed through a 400 mesh sieve according to Morris (1964).

The analytical ultracentrifugations were done on a Beckman Spinco E-HT instrument, at 20°C and 59 780 r.p.m. The analyzed protein samples were dialyzed for 24 hours against 0.5 M KCl in 0.02 M borate NaOH buffer, pH 8.2.

Results and Discussion

Characterization of the crude digest

The digest obtained by trypsin treatment at low ionic strength in the presence of Ca^{2+} (briefly "Ca-digest") cannot be separated into well defined fractions with the traditional procedure for preparation of meromyosins: e.g. after removal of Ca^{2+} and dialysis against 0.067 M phosphate buffer, pH 7, we have obtained a varying quantity of insoluble (i.e. LMM-like) material. However, if the protein which dissolved upon digestion was removed before the removal of Ca^{2+}, we obtained a fairly reproducible fractionation, the soluble fraction amounting to 20–30 per cent of the total with a reasonably constant specific ATP-ase activity. The soluble and insoluble protein fractions obtained in the presence of Ca^{2+} at the end of the digestion treatment were separately analyzed.

Due to the heterogeneous nature of the system, the reproducibility of the quantitative results is not as good as in the case of the traditional fragmentation.

Nevertheless, a few characteristics of the components of the digest are invariable: the intrinsic viscosity of the soluble protein is relatively low (0.09–0.30). The value of 2.5 for the intrinsic viscosity of the precipitate which was given in our previous paper (Biró et al., 1966) was not always obtained, however, it was never

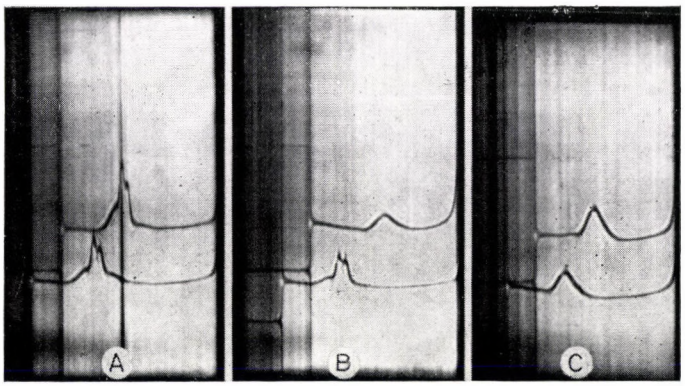

Fig. 1. Ultracentrifugal pattern of the crude "Ca digest" and of the two fractions. A: upper line: precipitated fraction; lower line: crude digest. Concentration of protein 6 mg/ml; 66 min after reaching full speed. B: Comparison of the precipitate and supernatant of the same digest. Upper line: supernatant 6 mg/ml; lower line: precipitate 3 mg/ml — 43 min after reaching full speed. C: Supernatant fraction — lower line: crude; upper line: after precipitation with 60 per cent ammonium sulphate. Concentration of protein 4 mg/ml; 44 min after reaching full speed. (For further experimental details see: "Methods")

lower than 2.0 in twelve experiments. Similarly, there were certain variations in the ultracentrifugal pattern of the crude digest and of the precipitate (compare Figs 1A, 1B, 4A, 4C), but these were of a quantitative rather, than of a qualitative character.

Figure 1A shows the ultracentrifugal pattern of a crude digest and of a precipitate fraction which has been repeatedly re-precipitated. The two patterns are very similar. On the other hand, the supernatant fraction (Fig. 1B, upper line), that is the protein dissolved in the course of digestion, is markedly different compared to the precipitate fraction (lower line). A single polydisperse component can be observed in the supernatant fraction without any boundary sharpening effect. The precipitate fraction shows the rather complex picture characteristic of the complete digest. Obviously, the soluble fraction is not discernible as a separate fraction in the ultracentrifugal pattern, since it only comprises 20–30 per cent of the total protein and its average sedimentation velocity ($S_{20} = 6.0$) does not differ considerably from the sedimentation velocity of the main components of the precipitate fraction. However, when the soluble proteins are isolated and concentrated, it is seen that the soluble fraction consists of distinctly different proteins as compared to the precipitate fraction. The components which show boundary

sharpening, indicating a relatively high molecular asymmetry, are only present in the precipitate.

Since the ultracentrifugal behaviour of the solubilized and undissolved proteins, respectively, was clearly distinct, we undertook further studies of the isolated fractions.

The supernatant fraction

The supernatant fraction has been re-precipitated by ammonium sulphate at 60 per cent saturation and dialyzed. This precipitation greatly diminished the contamination by a low molecular weight material, which did not separate from

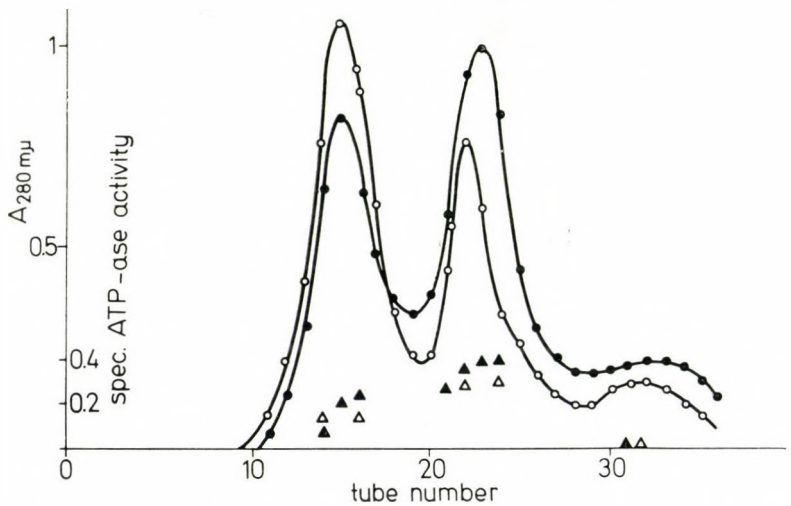

Fig. 2. Gel filtration pattern of the supernatant fraction. Abscissa: fraction number (5 ml fractions). Ordinate: optical density at 280 mμ (outer scale) and specific ATP-ase activity (inner scale, in units of μM P $min^{-1} \cdot mg^{-1}$). Circles: optical density, triangles: ATP-ase activity. Filled symbols: 20 min digest. Open symbols: 5 min digest

the meniscus (see Fig. 1C, lower line: "crude"; upper line: precipitated supernatant fraction). By this purification step the concentration of the impurity absorbing at 260 mμ was also considerably lowered.

The supernatant fraction could be resolved by gel-filtration on Sephadex G-200 into three fractions (Fig. 2, full circles). Two fractions which were present in roughly equal quantities could be identified as HMM, and HMM subfragment-1, respectively. The minimal molecular weight of the first fraction which is completely excluded from the gel is in the 300 000 – 400 000 range. Its specific ATP-ase activity corresponds to that of HMM. The molecular weight of the second fraction is about 170 000 on the basis of its elution volume, and its specific activity is about

1.5 times higher than that of the first fraction. This ratio corresponds to the ratio obtained by Mueller (1965) for HMM and subfragment-1. Calibrations with serum albumin and gamma-globulin support the above conclusions concerning the two molecular weights.*

The third fraction which is present in a low quantity shows no ATP-ase activity. Its elution volume practically equals the total volume of the column.

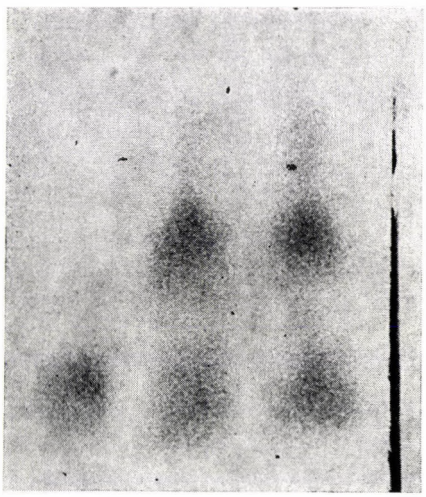

Fig. 3. Thin-layer gel-filtration pattern of the supernatant fraction. Left: HMM; middle: 5 min digest; right: 20 min digest. Direction of solvent flow downward

Conceivably, it consists of bigger peptides which precipitate at 60 per cent ammonium sulphate saturation and are not dialyzable.

According to the results of Biró and Fábián (unpublished) the amount of the supernatant fraction does not increase markedly when digestion is prolonged from 5 to 20 minutes. The composition of the supernatant obtained after 5 minutes of digestion is shown in Fig. 2 with open circles.

By comparing the two curves (see also the thin-layer chromatograms, Fig. 3) it is clear that the same fractions are present and that their ratio hardly changes: the relative amount of HMM decreases, and that of subfragment-1 increases between the 5th and 20th minute of digestion, but these changes are negligibly small.

By thin-layer gel-filtration of 5-minute and 20-minute supernatant of the "Ca digest" (Fig. 3) we obtained the same picture involving the same components.

* The identity of the second fraction with HMM subfragment-1 is indicated by the fact that the supernatant of the "Ca-digest" is routinely used in the laboratory of Bárány by Oppenheimer and co-workers (personal communication) as starting material for the production of subfragment-1.

The undissolved fraction

To identify the components of the precipitated fraction, we carried out experiments with the ultracentrifuge. Generally, we found two main components showing very low boundary diffusion and closely following each other. These

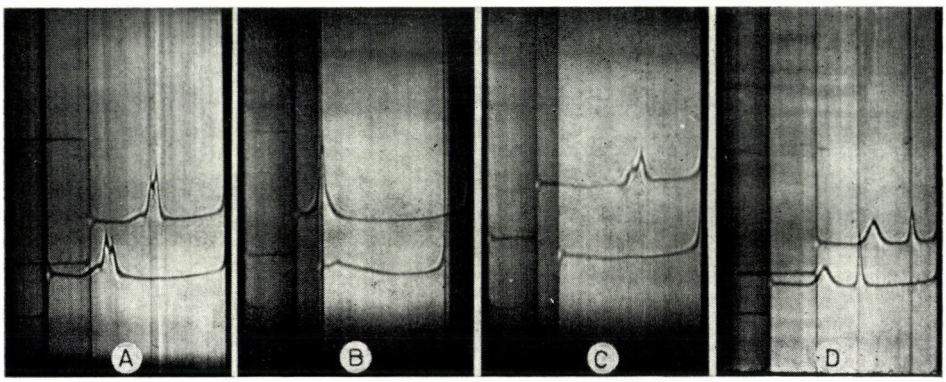

Fig. 4. Ultracentrifugal experiments for the identification of the components of the precipitate fraction. A: The effect of the addition of myosin. Lower line: control precipitate fraction, 4 mg/ml; upper line: with added myosin; to 3 mg/ml precipitate fraction 1 mg/ml myosin was added. 71 min after reaching full speed. B and C: The effect of the addition of actin. Upper line: control precipitate fraction, 4 mg/ml; lower line: to 5 mg/ml precipitate fraction 2.5 mg/ml purified F-actin was added. Pictures were taken at 25 (B) and 89 (C) min after reaching full speed. Experiments B and C were carried out with two different preparations. D: Mixtures of LMM + myosin. Upper line: 60 per cent LMM, lower line: 50 per cent LMM. Total protein concentration 4 mg/ml; 68 min after reaching full speed

markedly asymmetric components were followed by a smaller component with a diffuse boundary.

The one of the two main components showing the higher S_{20} value cannot be distinguished with hydrodynamic methods from myosin. As Fig. 4 shows, when undigested myosin is added to the precipitated fraction this clearly results in an increase in the amount of the faster component. Though we cannot directly prove that the component sedimenting with myosin is indeed intact myosin, we shall refer to it as myosin for the sake of convenience. The peak closely following the myosin peak indicates the formation of a hitherto unknown fragment which, on the basis of its sedimentation behaviour, can obviously be neither myosin (see above) nor HMM. In the course of our isolation procedure HMM appears in the supernatant and its S_{20} value is known to be always higher than that of myosin. At the same time this unknown component binds to actin as myosin and HMM do (Fig. 4B). Its binding at high ionic strength to actin excludes in itself the possibility of it being some LMM-like component. This is supported by the sedimentation behaviour of the small component left in solution after

the sedimentation of the components giving complexes with actin. This is evidently identical with the "trailing shoulder" appearing always in a low quantity in the precipitated fraction. Its S_{20} value which can be evaluated after removal of the actin complexes is about 3 (Fig. 4B). Accordingly, this small component should be identical with LMM-like components. It would further follow that the S_{20} value of the unknown component falls between those of LMM and myosin.

To exclude the possibility that the double peak present in the precipitated fraction is perhaps due to an LMM-myosin mixture of an unusual proportion, we also investigated the behaviour of LMM-myosin mixtures of different proportions. It can be seen in Fig. 4D that a perfect separation has been obtained in these experiments. Thus it is quite obvious that the peak in question contains a hitherto unknown myosin fragment. As for the ultracentrifugal behaviour of this material, its asymmetry approximates that of myosin and it binds to actin like myosin. However, it is certainly different from myosin.

In ultracentrifugal experiments with seven different digests, in which total protein concentration was varied between 3 and 6 mg/ml, we found that the S_{20} value of the new component was always lower by 0.5–0.7 Svedberg unit than that of myosin (6.4 S – Lowey, Holtzer, 1959). No definite concentration dependence of this difference can be observed which would exceed the experimental error of the procedure. Considering that the new component gives a hypersharp peak like myosin, it may be assumed that the concentration dependence of its sedimentation constant is very similar to that of myosin. On the basis of this consideration an intrinsic sedimentation constant of 5.6–5.8 can be tentatively attributed to it. A calculation based on the formula of Trautman et al. (1954) with $S^0_{20} = 5.7$ for the new component and with $S^0_{20} = 6.4$ for myosin will yield about 30 per cent "piling up" of the slower component due to the Johnson–Ogston effect.

Considering this, the quantity of the new component can be estimated from area measurement, and this yields a value of about 30–40 per cent of the total protein of the undissolved fraction.

In one of our foregoing papers (Biró et al., 1966) analyzing the mechanism of digestion at low ionic strength and in the presence of Ca^{2+}, we suggested that under such circumstances the "head" of myosin, HMM subfragment-1, can be split off directly, leaving behind the whole helical shaft. The comparison of the gel-filtration profile of the supernatant fraction obtained from the 5- and 20-minute digests, respectively (Figs 2 and 3) seems to indicate that subfragment-1 is split off (at least partly) directly from myosin. At the same time the unknown ultracentrifugal component observed in the Ca-digest cannot be identical with the postulated "beheaded" myosin molecule. It is easy to figure out with the Rieseman–Kirkwood equation (Rieseman, Kirkwood, 1950), that a rod-shaped molecule with a diameter of LMM and with an S^0_{20} approaching the value of 6.0 would have a molecular weight several times higher than myosin. If there really are molecules originating from the shaft of the myosin molecule but longer than LMM, these can only be present in the trailing shoulder having an S_{20} value near 3.0.

It would be certainly interesting to know by what kind of modification could have the component with $S_{20} = 5.6-5.8$ arisen from myosin? It is conceivable that the swelling of the globular parts of myosin is increased as a consequence of the proteolytic treatment without any considerable loss of weight, as it has been assumed by Kominz et al. (1965) to be the case with HMM obtained by papain digestion. An alternative explanation would involve a lowering of the molecular weight by some 50 000 – 60 000, with an insignificant change in the gross shape of the molecule. This latter assumption is based upon the myosin model, recently constructed by Stracker and co-workers (Dreizen et al., 1967), according to which there are three globular subunits with a molecular weight of about 20 000 in the "head" region of the myosin. They obtained (by dissociation at alkaline pH) a "heavy alkaline component" with a molecular weight of 430 000 and with an S_{20} of 5.6. Presumably, the "g-subunits" described by Dreizen et al. (1967) are lost during the proteolytic treatment which we have applied in our studies.

Both of these assumed structural changes could produce a molecule sedimenting but slightly slower than myosin. However, we should like to stress that a number of further explanations are equally possible. The relationship of the new component to the parent molecule has to be elucidated in further experiments.

Since this paper was sent to press Lowey et al. described (in: Symposium on Subunit Structure of Biological Macromolecules, Cambridge, 1968) that a double hypersharp peak, similar to that described here can be observed in early phases of the digestion of myosin with papain at low ionic strength. They identified the faster component as unchanged myosin while the slower one was suggested (on the basis of electronmicroscopic studies) to be a myosin molecule which lost one of the globular units as a result of proteolysis.

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Effect of Steric Changes in the Protein on the Kinetics of Enzymic Reactions

II. Steady-state Treatment of Reactions with One Substrate

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The theory and the equations of initial velocity of reactions with one substrate are elaborated, taking into account the steric changes in the enzyme and using the steady-state assumption.

In the case of cyclic models the initial rate of enzyme action is a quadratic function of substrate concentration. In these cases a sigmoid relationship between the reaction velocity and the substrate concentration may be obtained without any further assumption on the cooperative interaction of subunits.

In the case of non-cyclic models the initial rate of enzyme action is a hyperbolic function of substrate concentration, however, the maximum velocity and the Michaelis constant are the combination of the rate constant of the breakdown of the enzyme-substrate complex as well as of the formation and breakdown of this and of the rate constants of the preliminary elementary steps.

The equations of initial velocities presented give also the steady-state treatment of different types of inhibition and activation.

A general rule concerning the graphical evaluation of the equation of initial velocity of non-cyclic models is presented.

Ample experimental evidence confirmed during the last decade that some enzymes may undergo steric changes during catalysis. These steric changes may or may not influence the kinetics of the reaction.

This paper deals with the possibilities when the case of steric changes in the enzyme may be:

a) the binding of the substrate – induced fit theory (Koshland, 1960);

b) an inherent property of the protein – its motility (Linderstrøm-Lang, Schellman, 1959). In this case the binding of the substrate may stabilize one of the different conformations which are in dynamic equilibrium with each other (Straub, Szabolcsi, 1964).

In both cases the steric changes in the enzyme are called:

a) essential for the reaction when only the changed enzyme* is able to catalyze the formation or the breakdown of the enzyme-substrate complex;

* "Enzyme" (E) means a population of enzyme molecules which do not suffer such steric changes which influence their kinetic properties. "Changed enzyme" (E') means a population of enzyme molecules which underwent such steric changes which influence their kinetic properties. This differentiation is unvalid only if in the equilibrium $E \rightleftarrows E'$ the two forms

b) non-essential, when the enzyme or both the enzyme and the changed enzyme are able to catalyze the formation and the breakdown of the enzyme-substrate complex.

The theory, the equations of initial velocity, the general equation and the Haldane relationship of reactions with one substrate were elaborated, assuming that the steric changes in the enzyme influence essentially or non-essentially the rate of enzyme activity, when one of the elementary steps is assumed to be rate limiting (Keleti, 1967).

In the case of the steady-state treatment of similar processes the number of kinetic constants greatly increases when the system becomes more complicated and it becomes impossible to determine experimentally all the constants involved in the equation. However, it is useful to obtain the equations for the rates as a function of substrate concentration since the general features of these equations give valuable informations on the nature of such processes.

Equations of initial velocity

Cyclic models

The equations of initial velocity of cyclic models were solved using the graphical method of King and Altman (1956), simplified by Volkenstein (1965), Volkenstein and Goldstein (1966, 1966a).

In all cases: E = enzyme, E' = changed enzyme, E_T = total enzyme, S = substrate, P = product.

The general mechanism* which involves all possibilities of essential and non-essential steric changes in the enzyme which affect the catalytic activity is presented in Fig. 1.

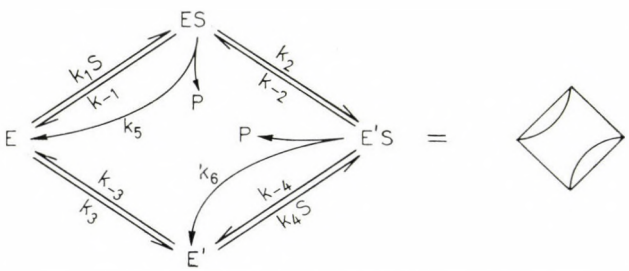


Fig. 1. Scheme of the general mechanism

of the enzyme are present in equimolar amounts (50—50 per cent, i.e. $k_3 = k_{-3}$). If this restriction is not taken into account, one of the two forms must be in a higher concentration than the other. In this case the form occurring in a higher concentration is considered as "enzyme". The possibility of more enzyme forms with different kinetic properties (e.g. E'', E''' etc.) is not discussed in this paper.

* Some properties (e.g. energetics, thermodynamics) of a similar model (but with some

The different possibilities of essential or non-essential steric changes in the enzyme are summarized in Table 1.

Table 1

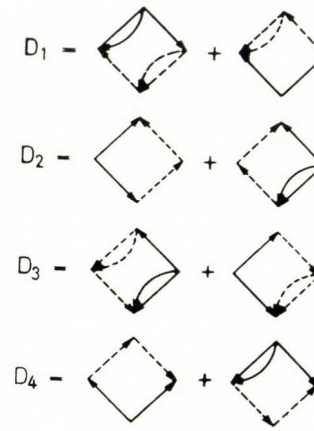
Essential or non-essential steric changes in the enzyme during catalysis

The models marked with I. mean essential steric changes and those marked with II. mean non-essential steric changes in the enzyme

The substrate forms complex with	The breakdown of the enzyme-substrate complex is catalyzed by		
	the enzyme	the changed enzyme	both the enzyme and the changed enzyme
the enzyme	II.a.1.α.  II.a.1.β.  II.a.1.γ.  II.a.1.δ. 	I.b.2. 	II.a.2. 
the changed enzyme	I.a.2. 	I.a.1.α.  I.a.1.β. 	I.a.3. 
both the enzyme and the changed enzyme	II.b.1.α.  II.b.1.β. 	I.b.3.α.  I.b.3.β. 	II.b.2.α.  II.b.2.β. 

The models II.b.1.α, I.b.3.β. and II.b.2.β. are cyclic models. The determinants are solved in the general case (II.b.2.β.) as follows:

restrictions) are discussed in the paper of Jencks (1966), without presenting the equations of different possible reaction types. The general model, in the case of rapid equilibrium if the step $ES \rightleftharpoons E'S$ is the rate limiting one and if the reversibility of some steps is not complete, gives sigmoid relationship between the initial velocity and the substrate concentration as discussed by Rabin (1967).



where the dotted arrow multiplied by dotted arrow, multiplied by the sum of arrows gives the "numerical" value of the determinant (Volkenstein, Goldstein, 1966a) and the arrows mean the kinetic constant or the product of kinetic constant and substrate concentration shown in Fig. 1.

In other cyclic cases the arrows of non functioning elementary steps are omitted in the graphical evaluation of the determinants.

The values of the determinants and the equations of initial velocity of cyclic models are summarized in Table 2.

The form of $v = f(S)$ of cyclic models show that a quadratic relationship between the initial velocity and the substrate concentration does not show unequivocally the binding of two substrate molecules on the enzyme. On the other hand, sigmoid or other quadratic relationship between the initial reaction velocity and the concentration of one substrate (if the other is maintained constant) may be obtained also in the case of reactions with two substrates if the mechanism of the reaction is random and the kinetics is steady-state and in some other cases (Segal et al., 1952; Kistiakowsky, Rosenberg, 1952; Alberty, 1953; Alberty, Bock, 1953; Alberty et al., 1954; Ingraham, Makower, 1954; King, 1956; King, Altman, 1956; Dalziel, 1958; Botts, 1958; Wong, Hanes, 1962; Ferdinand, 1966). Therefore with pure kinetic methods it is not possible to distinguish between these mechanisms or between those where steric changes in the enzyme occur as well as where cooperative interaction between subunits takes place.

Non-cyclic models

The general rule of the graphical evaluation of the equation of initial velocity of non-cyclic models is as follows:

a) the numerator is the product of the rate constant of the end-product (P) formation, of total enzyme concentration and of rate constants represented by the arrows all ending in that enzyme-substrate complex which gives the end-product;

b) the denominator is the sum of the products of rate constants represented by the arrows ending in each enzyme-substrate complex (including the free enzyme). It is to be noted that parallel arrows (e.g. ) represent a sum of the corresponding rate constants as in the evaluation of cyclic models (see: Volkenstein, Goldstein, 1966a).

c) calculating both numerator and denominator, the number of arrows to be used is given by the number of elementary steps in the model, excluded the formation of end-product (if this does not give parallel arrows [e.g. ]).

Table 3 summarizes the equations of initial velocity for non-cyclic models – presented with the method of graphs – and the maximum velocities as well as the Michaelis constants.

It is to be noted that in the cases of non-cyclic models the form of $v = f(S)$ is always a hyperbola. However, the meaning of V_{max} and K_M in the general equation $v = V_{max}[S]/(K_M + [S])$ is a much more complicated function of rate constants as in the original Briggs–Haldane equation (Briggs, Haldane, 1925) represented by the model II.a.1.α.

Inhibition and activation types

The graphical evaluation of different cyclic and non-cyclic models gives at the same time the steady-state treatment of different inhibition and activation types.*

If I = *inhibitor*, $E' = EI$, instead of k_3 we write $k_3 I$ and instead of k_2 we write $k_2 I$ then II.b.1.α gives the steady-state treatment of mixed type of inhibition (since purely non-competitive inhibition is impossible in steady-state systems – see: Morales, 1955) and II.b.2.β that of partially non-competitive or partially competitive inhibition. II.a.2 gives the steady-state treatment of apparently mixed type of inhibition – if IE is an inactive complex (Keleti, Telegdi, 1966) –, II.a.1.β that of purely uncompetitive inhibition, I.a.1.β that of apparently uncompetitive inhibition (Keleti, Telegdi, 1966) and II.a.1.γ that of purely competitive inhibition.**

If A = activator, $E' = EA$, instead of k_3 we write $k_3 A$ and instead of k_2 we write $k_2 A$ then I.a.1.α, I.b.2, I.b.3.α and I.b.3.β show the steady-state treatment of different activation types.

The models presented in this paper do not give the steady-state treatment of different "liberation" types (Keleti, 1967a).

Acknowledgement

The valuable discussions of Dr J. Südi are gratefully acknowledged.

* The steady-state treatment of different types of inhibition see: Botts and Morales (1953), Hearon et al. (1959), Walter (1962) and Frieden (1964).

** The steady-state treatment of purely competitive inhibition gives the same result as the rapid equilibrium treatment — see: Dixon and Webb (1964), Webb (1963) and Gutfreund (1965).

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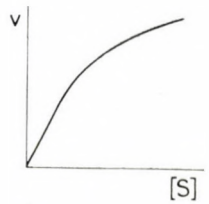
Table 2

Equations of initial velocity of cyclic models

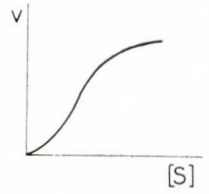
It is to be noted that I.b.3.β and II.b.1.α are mirror images of each other

Mechanism	Graphical presentation	Determinants	Initial velocity	form of $v = f(S)$
I.b.3.β		$D_1 = k_{-3}(k_{-4} + k_6)(k_{-1} + k_2) + k_{-2}k_{-1}(k_{-3} + k_4[S])$ $D_2 = k_4 k_{-2}[S](k_1[S] + k_3) + k_{-3}k_1[S](k_{-4} + k_6 + k_{-2})$ $D_3 = k_{-1}k_3(k_{-4} + k_6 + k_{-2}) + k_2(k_{-4} + k_6)(k_1[S] + k_3)$ $D_4 = k_1 k_2[S](k_{-3} + k_4[S]) + k_3 k_4[S](k_{-1} + k_2)$	$v = \frac{k_6 D_4 [E]_T}{D_1 + D_2 + D_3 + D_4}$	$v = \frac{a[S] + b[S]^2}{c + d[S] + e[S]^2}$ quadratic equation*
II.b.1.α		$D_1 = k_{-3}k_{-1}(k_{-1} + k_5 + k_2) + k_{-2}(k_{-1} + k_5)(k_{-3} + k_4[S])$ $D_2 = k_1 k_{-2}[S](k_1[S] + k_3) + k_1 k_{-3}[S](k_{-4} + k_{-2})$ $D_3 = k_3(k_5 + k_{-1})(k_{-4} + k_{-2}) + k_2 k_{-4}(k_1[S] + k_3)$ $D_4 = k_1 k_2[S](k_{-3} + k_4[S]) + k_3 k_4[S](k_{-1} + k_5 + k_2)$	$v = \frac{k_5 D_2 [E]_T}{D_1 + D_2 + D_3 + D_4}$	as in I.b.3.β
II.b.2.β		$D_1 = k_{-3}(k_{-4} + k_6)(k_{-1} + k_5 + k_2) + k_{-2}(k_{-1} + k_5)(k_{-3} + k_4[S])$ $D_2 = k_4 k_{-2}[S](k_1[S] + k_3) + k_1 k_{-3}[S](k_{-4} + k_6 + k_{-2})$ $D_3 = k_3(k_5 + k_{-1})(k_{-4} + k_6 + k_{-2}) + k_2(k_{-4} + k_6)(k_1[S] + k_3)$ $D_4 = k_1 k_2[S](k_{-3} + k_4[S]) + k_3 k_4[S](k_{-1} + k_5 + k_2)$	$v = \frac{(k_5 D_2 + k_6 D_4)[E]_T}{D_1 + D_2 + D_3 + D_4}$	as in I.b.3.β

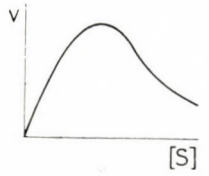
* If $[S] \to \infty$, $v \equiv V_{max} \to$ b/e. According to Botts (1958) the form of the curve v vs $[S]$ may be:



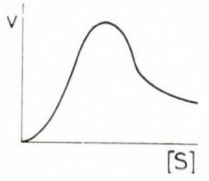
if $bd > ae, bc > 0, ac > 0, ad > bc$



if $bd > ae, bc > 0, ac > 0, bc > ad$



if $ae > bd, bc > 0, ac > 0, ad > bc$

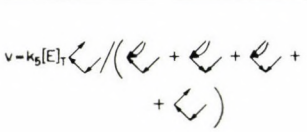


if $ae > bd, bc > 0, ac > 0, bc > ad$

Table

Equations of the initial velocity

It is to be noted that II.a.1.γ and I.a.1.α, II.a.1.δ and I.a.1.β, II.b.1.β and I.b.3.α, I.b.2. (see footnote on page 247) the Michaelis constant of models I.a.1.α, I.a.1.β, I.b.3.α, II.a.1.γ, or the maximum velocity does not depend any more

Mechanism	Graphical presentation	Equation of initial velocity presented with the method of graphs
I.a.1.α.		
I.a.1.β.		
I.b.3.α.		
II.a.1.α. (Briggs-Haldane kinetics)		
II.a.1.β.		
II.a.1.γ.		
II.a.1.δ.		
II.b.1.β.		

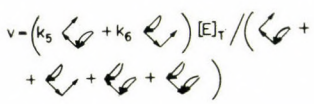
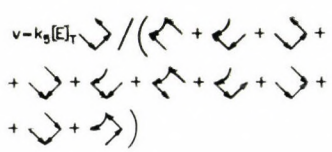
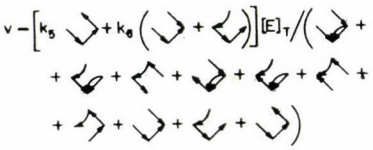
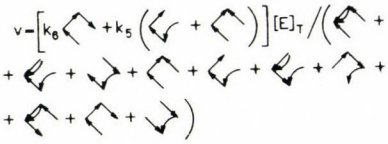
3

of non-cyclic models

and I.a.2., I.a.3. and II.a.2. are mirror images of each other. If $[E] = [E']$, i.e. $k_3 = k_{-3}$ II.a.1.δ, II.b.1.β and II.b.2.α will have a factor of two, but either the Michaelis constant on the rate constants of $E \rightleftarrows E'$ equilibrium.

V_{max}	K_M
$k_6 [E]_T$	$\frac{(k_{-1} + k_6)(k_3 + k_{-3})}{k_3 k_1}$
$\frac{k_2 k_6 [E]_T}{k_2 + k_{-2}}$	$\frac{k_2 (k_{-1} + k_6)(k_{-3} + k_3)}{(k_2 + k_{-2}) k_3 k_1}$
$\frac{k_6 k_{-1} k_3 k_1 [E]_T}{(k_{-1} + k_6) k_1 k_{-3} + k_{-1} k_3 k_1}$	$\frac{k_{-1} (k_{-1} + k_6)(k_{-3} + k_3)}{(k_6 + k_{-1}) k_1 k_{-3} + k_{-1} k_3 k_1}$
$k_5 [E]_T$	$\frac{k_{-1} + k_5}{k_1}$
$\frac{k_{-2} k_5 [E]_T}{k_2 + k_{-2}}$	$\frac{k_{-2} (k_{-1} + k_5)}{k_1 (k_2 + k_{-2})}$
$k_5 [E]_T$	$\frac{(k_{-1} + k_5)(k_3 + k_{-3})}{k_1 k_{-3}}$
$\frac{k_{-2} k_5 [E]_T}{k_2 + k_{-2}}$	$\frac{k_{-2} (k_{-1} + k_5)(k_3 + k_{-3})}{(k_2 + k_{-2}) k_1 k_{-3}}$
$\frac{k_5 k_1 k_{-3} k_{-4} [E]_T}{(k_{-1} + k_5) k_3 k_4 + k_1 k_{-3} k_{-4}}$	$\frac{k_{-4} (k_{-1} + k_5)(k_3 + k_{-3})}{(k_5 + k_{-1}) k_3 k_4 + k_1 k_{-3} k_{-4}}$

Table 3 (continued)

Mechanism	Graphical presentation	Equation of initial velocity presented with the method of graphs
II.b.2.α.		
I.b.2.		
I.a.2.		
I.a.3.		
II.a.2.		

$V_{\max}$	K_M
$\dfrac{[k_5 k_{-3} k_1 (k_{-4} + k_6) + (k_{-1} + k_5) k_6 k_3 k_4] [E]_T}{(k_5 + k_{-1}) k_3 k_4 + k_{-3} k_1 (k_6 + k_{-4})}$	$\dfrac{(k_{-1} + k_5)(k_{-4} + k_6)(k_{-3} + k_3)}{k_{-3} k_1 (k_{-4} + k_6) + k_3 k_4 (k_{-1} + k_5)}$
$\dfrac{k_6 k_2 k_{-3} [E]_T}{k_2 k_6 + k_{-2} k_{-3} + k_{-3} k_6 + k_2 k_{-3}}$	$\dfrac{(k_3 + k_{-3})(k_{-1} k_{-2} + k_{-1} k_6 + k_2 k_6)}{k_1 (k_2 k_6 + k_{-2} k_{-3} + k_{-3} k_6 + k_2 k_{-3})}$
$\dfrac{k_5 k_{-2} k_3 [E]_T}{k_3 k_{-2} + k_3 k_5 + k_2 k_3 + k_5 k_{-2}}$	$\dfrac{(k_3 + k_{-3})(k_5 k_{-2} + k_5 k_{-4} + k_2 k_{-4})}{k_4 (k_3 k_{-2} + k_3 k_5 + k_2 k_3 + k_5 k_{-2})}$
$\dfrac{[k_5 k_{-2} k_3 + k_6 (k_2 k_3 + k_3 k_5)] [E]_T}{k_{-2} k_5 + k_2 k_3 + k_3 k_5 + k_{-2} k_3}$	$\dfrac{(k_3 + k_{-3})(k_2 k_{-4} + k_{-4} k_5 + k_{-2} k_5 + k_2 k_6 + k_5 k_6)}{(k_2 k_3 + k_{-2} k_5 + k_3 k_5 + k_{-2} k_3) k_4}$
$\dfrac{[k_6 k_2 k_{-3} + k_5 (k_{-2} k_{-3} + k_{-3} k_6)] [E]_T}{k_2 k_6 + k_{-2} k_{-3} + k_{-3} k_6 + k_2 k_{-3}}$	$\dfrac{(k_3 + k_{-3})(k_{-2} k_{-1} + k_{-1} k_6 + k_2 k_6 + k_{-2} k_5 + k_5 k_6)}{(k_2 k_6 + k_{-2} k_{-3} + k_{-3} k_6 + k_2 k_{-3}) k_1}$

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Mechanism of the Action and of the Allosteric Inhibition of 3-Deoxy-D-arabino-heptulosonate 7-Phosphate Synthase (Tyrosine-sensitive) of Escherichia Coli W

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1. The tyrosine-sensitive allosteric first enzyme (isoenzyme 1b) of the aromatic amino acid biosynthetic pathway, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (7-phospho-2-oxo-3-deoxy-D-arabino-heptonate D-erythrose-4-phosphate-lyase [pyruvate phosphorylating]), E.C. 4.1.2.15, has been purified from Escherichia coli W.

2. The reaction proceeds at maximal rate over a broad range of pH between 6.0 and 7.5 in Tris-buffer. Optimum pH in phosphate-buffer is 6.3. The initial velocity data obtained obey normal Michaelis—Menten kinetics without any detectable kinetic evidence for subunit interaction. On the basis of kinetic experiments the mechanism of enzyme action is "Ping-Pong" and the "first" substrate is phosphoenolpyruvate. The "absolute" Michaelis constant of the enzyme for both phosphoenolpyruvate and erythrose 4-phosphate is 1.0×10^{-3} M.

3. L-tyrosine inhibits the activity of enzyme allosterically over a broad range of pH between 6.0 and 7.5 in Tris-buffer. The initial velocity data in the presence of tyrosine are compatible with regular Michaelis—Menten kinetics and the presence of tyrosine does not induce any kinetically detectable change in subunit interaction. The kinetic relationship between phosphoenolpyruvate and L-tyrosine is competitive; between erythrose 4-phosphate and L-tyrosine it is uncompetitive. For the competitive inhibition $K_{i\ \mathrm{slope}}$ is 4.5×10^{-6} M and for the uncompetitive inhibition $K_{i\ \mathrm{intercept}}$ is 2.0×10^{-5} M. Because of the mechanism of enzyme action and of inhibition $K_{i\ \mathrm{slope}}$ is equal to K_i.

4. Potassium cyanide and ethylenediaminetetraacetate inhibit, whereas after EDTA treatment Co^{2+} activates the enzyme. Co^{2+} protects the enzyme against the inactivating effect of alkaline pH and low concentrations (0.1 M) of urea. Para-hydroxy-mercuribenzoate inhibits the activity of enzyme also. According to the results obtained the enzyme is a Class II aldolase.

Introduction

The first step in the biosynthesis of the aromatic amino acids, of p-amino-benzoic acid and of p-hydroxybenzoic acid is the formation of 3-deoxy-D-arabino-heptulosonate 7-phosphate from erythrose 4-phosphate and phosphoenolpyruvate

Abbreviations: DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; E4P, erythrose 4-phosphate; PEP, phosphoenolpyruvate; EDTA, ethylenediaminetetraacetate; p-HMB, p-hydroxymercuribenzoate.

(Srinivasan, Sprinson, 1959). Three distinct isoenzymes of 7-phospho-2-oxo-3-deoxy-D-arabino-heptonate D-erythrose 4-phosphate-lyase (pyruvate phosphorylating), EC 4.1.2.15, referred to hereafter as DAHP synthase, which catalyze the formation of DAHP were detected in Escherichia coli W (Smith et al., 1962; Doy, Brown, 1965). The activity of isoenzyme 1a and 1b is inhibited allosterically by phenylalanine and tyrosine, respectively. The activity of isoenzyme 1c is not inhibited by any of the endproducts (Doy, Brown, 1965).

The present paper describes kinetic experiments on the mechanism of action and of inhibition of the purified tyrosine-sensitive DAHP synthase from E. coli.

Materials and Methods

Chemical. The inorganic chemicals used were of A.R. grade. L-tyrosine, L-phenyl-alanine, phosphoenolpyruvate, glucose 6-phosphate were obtained from "Reanal", Budapest. Thiobarbituric acid was purchased from Fluka A.G., Buchs. Shikimic acid was obtained from "Calbiochem".

Erythrose 4-phosphate was prepared from D-glucose 6-phosphate (Ballou, 1963), and purified by ion-exchange chromatography as described previously (Łobarzewski, Dénes, 1966).

Organisms and growth conditions. Wilde-type strain of Escherichia coli W and the mutant 83 – 1 (5-dehydrokinase less) were provided by Dr B. D. Davis. Minimal medium A containing 0.2 per cent of glucose was used (Davis, Mingioli, 1950). Since the level of the tyrosine-sensitive isoenzyme of DAHP synthase in the wild-type E. coli W growing on minimal medium is very low, we have used the mutant strain 83 – 1 for the isolation of the enzyme (Doy, Brown, 1965). Cells of the mutant strain 83 – 1 were grown on medium A containing 0.2 per cent of glucose and limiting amount, 40 μg per ml of shikimic acid. All cultures were grown at 37°C on a gyrotory shaker. Growth was followed turbidimetrically by sampling and reading the absorbance at 490 mμ in a 1 cm cuvette.

Enzyme assay. The crude extract of E. coli W 83 – 1 grown on limiting amount of shikimic acid and harvested in the stationary phase contains all three isoenzymes of DAHP synthase. For determination of the activity of the individual isoenzymes in the mixture we have used the following method. After determination of the overall activity of DAHP synthase in the crude extract, the residual activity measured in the presence of 1 mM phenylalanine and 1 mM tyrosine was considered as isoenzyme 1c. The decrease of overall activity of DAHP synthase in the presence of 1 mM phenylalanine was considered as isoenzyme 1a, and the decrease in the presence of 0.01 mM tyrosine as 1b. From the point of view of the determination of the activity of isoenzyme 1a and 1b in a mixture it is important to remember that phenylalanine inhibits the activity of isoenzyme 1a only, while tyrosine inhibits the activity of both isoenzymes 1b and 1a in concentrations higher than 0.1 mM (Doy, Brown, 1965).

Unless otherwise stated, the following standard reaction mixture was used: 20 μmoles of Tris-maleate buffer (pH 6.4), 0.50 μmole of phosphoenolpyruvate, 0.40 μmole of erythrose 4-phosphate and the enzyme in a final volume of 1.0 ml. The reaction was started by the addition of enzyme and the mixtures were incubated at 37°C. The reaction was arrested by the addition of 0.4 ml of 10 per cent trichloroacetic acid and the precipitated proteins were removed by centrifugation. The amount of DAHP formed was determined by the thiobarbituric acid method (Srinivasan, Sprinson, 1959). The method was slightly modified by decreasing the reaction time of DAHP with periodic acid to 30 minutes.

One unit of enzyme is defined as the amount that catalyzes the formation of 0.1 μmole of DAHP in 5 minutes.

Specific activity is expressed as units per mg of protein.

Protein was determined by the method of Lowry et al. with crystalline bovine serum albumine as a standard (Lowry et al., 1951).

The unit of the initial rate of reaction, v_0, is defined as 0.1 μmole of DAHP formed per 10 min per 1.0 ml of the reaction mixture. The unit of the initial velocity of the reaction in the presence of inhibitor, v_i, is defined in the same way. Inhibition (i) is expressed as $1 - v_i/v_0$.

Results and Discussion

Purification of the enzyme

Step 1. Preparation of crude extract. 8 liters of a culture of the mutant strain 83 – 1 were harvested by centrifugation at 0°C after two hours in stationary phase because of the exhaustion of the shikimic acid supplied. The sedimented cells were washed twice with 0.04 M potassium phosphate buffer (pH 7.4) and resuspended in 130 ml of the same buffer. The cells were disrupted by sonic treatment in a M.S.E. 20 kc oscillator for 10 min with cooling in an ice-water bath. Whole cells and cell debris were removed by centrifugation at 20 000 *g* for 20 min at 0°C.

Step 2. Fractionation with ammonium sulfate. The crude extract (128 ml) obtained in Step 1 was treated with solid ammonium sulfate to a saturation of 0.35. After the inactive precipitate was removed by centrifugation, the addition of ammonium sulfate was continued to a saturation of 0.42. The precipitate was dissolved in 12 ml of 0.04 M potassium phosphate buffer (pH 7.4) and the solution was dialyzed overnight at 0°C against the same buffer.

Step 3. Adsorption on calcium phosphate gel. The dialyzed solution, containing 212 mg of protein was diluted with 3 vol. of 0.1 mM $CoCl_2$ solution containing 0.2 mM tyrosine. The diluted enzyme was treated with 28 ml of calcium phosphate gel (151 mg dry weight per ml) at 0°C. After 10 minutes the mixture was centrifuged and the gel washed with 0.01 M potassium phosphate buffer (pH 6.4) containing 0.2 mM tyrosine and 0.1 mM $CoCl_2$. The enzyme was eluted with successive 6 ml portions of 0.04 M potassium phosphate buffer (pH 6.4) containing 0.2 mM tyrosine and 0.1 mM $CoCl_2$. The peak of the activity of the enzyme (with

maximum specific activity) was found in the second and third fractions. These fractions were pooled. The enzyme in the pooled fraction was precipitated by the addition of ammonium sulfate (0.5 saturation) and the precipitated enzyme was dissolved in 6 ml of 0.04 M potassium phosphate buffer.

Table 1

Summary of the purification procedure

Step	Fraction	Volume ml	Total units	Total protein mg	Specific activity	Yield %
1	Crude extract	128	2330	1792	1.3	100
2	Ammonium sulfate fractionation	12	2496	212	11.7	100
3	Calcium phosphate gel adsorption	6.2	1261	54	23.3	51

The partially purified enzyme obtained in Step 2 is stable and it is possible to store it for 5–10 days at −20°C without detectable loss of activity. The enzyme after the calcium phosphate gel treatment (Step 3) is very unstable and could not be stored for more than 12 hours. The kinetic experiments presented in this paper were performed with partially purified enzyme obtained in Step 2 after activation by freezing overnight.

The pH dependence of the reaction rate and of tyrosine inhibition

As illustrated in Fig. 1a the pH optimum for the synthase reaction in Tris-buffer is very broad, ranging from 6.0 to 7.5. The activity of the enzyme falls rapidly when the pH is lowered below pH 5.5 and gradually when the pH is increased from 7.5 to 8.5. A pH of 6.4 was chosen for the standard assay of the enzyme. The pH optimum of the catalytic reaction is rather sharp in phosphate buffer (pH 6.3) and it falls rapidly in both directions. In phosphate buffer containing 0.5 mM $CoCl_2$ the pH dependence of the rate of the reaction becomes very similar to that in Tris-buffer. Co^{2+} activates the enzyme in Tris-buffer but the shape of the curve, i.e. the pH dependence of the reaction rate remains the same.

As Fig. 1b shows, the pH dependence of inhibition of the enzyme activity is the same as that of the reaction velocity and depends similarly on the chemical nature of the buffer used. It is interesting to note that the inhibitory effect of tyrosine increases with increasing concentration of phosphate buffer, while the pH-optimum of inhibition is unchanged.

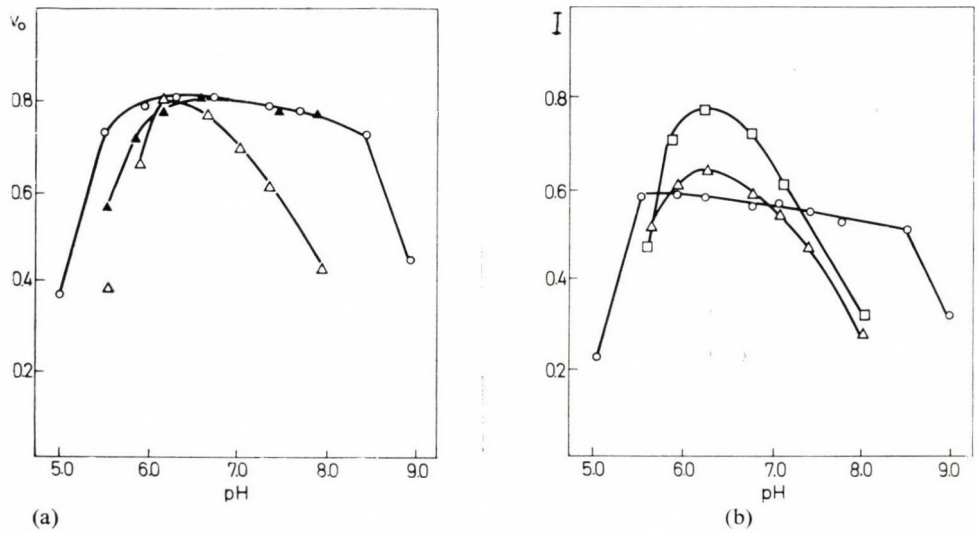


Fig. 1. pH dependence of the initial rate of DAHP formation (a) and of allosteric inhibition (b). Standard reaction mixture was used except for buffer containing 60 μg of protein (a) and the same reaction mixture containing 0.1 μmole of tyrosine for the determination of inhibition (b). ○——○, 0.02 M Tris-maleate, Tris-HCl buffer; △——△, 0.01 M potassium phosphate buffer; ▲——▲, 0.01 M potassium phosphate buffer containing 0.5 mM $CoCl_2$; □——□, 0.08 M potassium phosphate buffer. Inhibition (i) is expressed as $1 - v_i/v_0$

Inhibition and activators

The purified enzyme is active without the addition of any cofactor or ion. The activity of the freshly purified enzyme (Step 2) increases by about 50 per cent after freezing overnight at −20°C and this activation process is going on slowly up to a maximum value of 100 per cent in frozen state. At the sixth to seventh day the activity of the enzyme is about twice as high as that of the freshly prepared enzyme. As shown in Table 2, Co^{2+} activates the enzyme and the optimum concentration for the activation is 1 mM. The activity of the freshly prepared enzyme (Step 2) is about twofold in the presence of 1.0 mM Co^{2+}. The activating effect of Co^{2+} depends on the age of the frozen enzyme and decreases as the activity of the enzyme increases, because of the "cold" activation, as shown in Table 2. At the sixth-seventh day Co^{2+} does not activate the enzyme. EDTA inhibits the activity of the enzyme reversibly. After EDTA treatment Co^{2+} completely restores the activity of the enzyme as shown in Table 3. Other ions, as Ni^{2+}, Fe^{2+}, Zn^{2+}, Mg^{2+} are either slightly active or completely inactive in these experiments (Table 3). Potassium cyanide is a potent inhibitor of the enzyme and overnight dialysis against 1 mM KCN causes irreversible inactivation. It seems probable that the enzyme contains firmly bound heavy metal and belongs to the Class II aldolases (Rutter, 1964). The activity of the enzyme is inhibited by p-HMB also, as shown in Table 3.

Table 2

Effect of freezing and of Co^{2+} on the activity of DAHP synthase

For the determination of the initial rate the standard reaction mixture containing 170 μg of protein was used. The activity of the partially purified enzyme (Step 2) was determined in the absence and in the presence of 1.0 mM $CoCl_2$ either immediately after preparation or after freezing at —20°C as indicated

Exp.	Enzyme	Addition	v_0
1	Freshly prepared	0	0.42
		Co^{2+}	0.80
2	After freezing overnight	0	0.60
		Co^{2+}	0.79
3	After freezing for 6 days	0	0.80
		Co^{2+}	0.81

Table 3

Effect of inhibitors and activators on the initial rate of DAHP synthase

For the determination of the initial rate the standard assay mixture was used. The enzyme 170 μg per ml in Tris-maleate buffer (pH 6.5) was incubated in the presence of inhibitor at 37° for 10 min. The assay was initiated by the addition of substrates. In Expts 2—8 the enzyme was incubated with EDTA for 10 min. at 37°, then after addition of the bivalent ion studied, the assay was initiated immediately by the addition of the substrates. In Expt 10 the enzyme (1.2 mg per ml) in Tris-maleate buffer (pH 6.5) was incubated at 37° for 4 min. in the presence of 0.1 M urea, then it was diluted tenfold in the same buffer containing p-HMB. After incubation for 10 min. at 37° the assay was initiated by the addition of substrates

Exp.	Addition	v_0
1	none	0.60
2	0.1 mM $CoCl_2$	0.78
3	0.01 mM EDTA	0.17
4	0.01 mM EDTA + 0.1 mM $CoCl_2$	0.79
5	0.01 mM EDTA + 0.1 mM $NiCl_2$	0.53
6	0.01 mM EDTA + 0.1 mM $FeSO_4$	0.50
7	0.01 mM EDTA + 0.1 mM $ZnCl_2$	0.14
8	0.01 mM EDTA + 0.1 mM $MgCl_2$	1.15
9	3.6×10^{-6} M p-HMB	0.10
10	3.6×10^{-6} M p-HMB + 0.1 M urea	0.10
11	8.0×10^{-8} M p-HMB	0.60
12	8.0×10^{-8} M p-HMB + 0.1 M urea	0.10

Initial velocity pattern

The dependence of the initial velocity of DAHP formation on PEP concentration in the presence of nearly saturating concentrations of E4P follows the regular Michaelis—Menten kinetics. The initial rate of DAHP formation in the

presence of saturating concentrations of PEP and various concentrations of E4P follows the regular Michaelis–Menten kinetics also without any detectable kinetic evidence for subunit interaction, as presented in Figs 2 and 3.

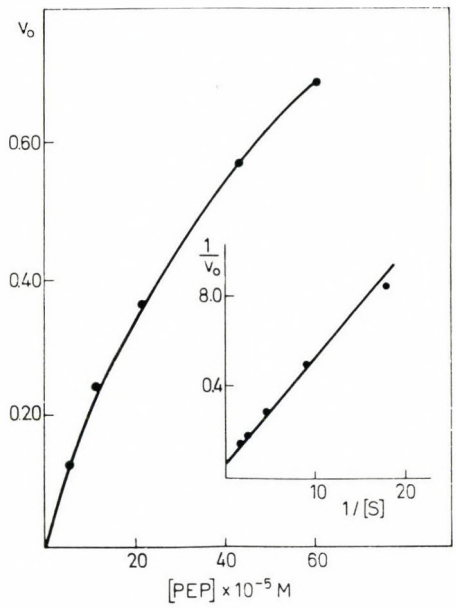


Fig. 2. Effect of phosphoenolpyruvate concentration on the initial rate of DAHP formation. The reaction mixture contained 20 μmoles of Tris-maleate buffer (pH 6.5), 0.40 μmole of erythrose 4-phosphate, 60 μg of protein and phosphoenolpyruvate as indicated in a total volume of 1.0 ml

We have previously found that the mechanism of action of the phenylalanine sensitive DAHP synthase (isoenzyme 1a) is "Ping-Pong" (Staub, Dénes, 1967). It was reasonable to suppose that the mechanism of action of the tyrosine-sensitive enzyme was similar. As shown in Fig. 4 the plot of the reciprocal of the initial rate of DAHP formation against the reciprocal of PEP concentration at four fixed concentrations of E4P gives a set of parallel lines. It can be seen that there is apparently no tendency for the curves to intersect at a common point to the left of the ordinate. The plots of the reciprocal of E4P concentration against the reciprocal of the initial rate of DAHP formation at four fixed concentrations of PEP give a set of parallel lines also. The intercepts on the ordinate are the reciprocals of the apparent maximal velocities $1/V'$ and the intercepts on the abscissa are negative reciprocals of the apparent Michaelis constants, $-1/K_m'$. The "absolute" Michaelis constants of the enzyme for E4P (K_{E4P}) and for PEP (K_{PEP}) are determined graphically from secondary plots of the data shown in Fig. 4.

As Fig. 5 shows the absolute Michaelis constant of the enzyme for both PEP and E4P is 1.0×10^{-3} M. The mechanism of reaction appears to be "Ping-Pong" (Cleland, 1963a). This mechanism indicates that the mechanism of enzyme and

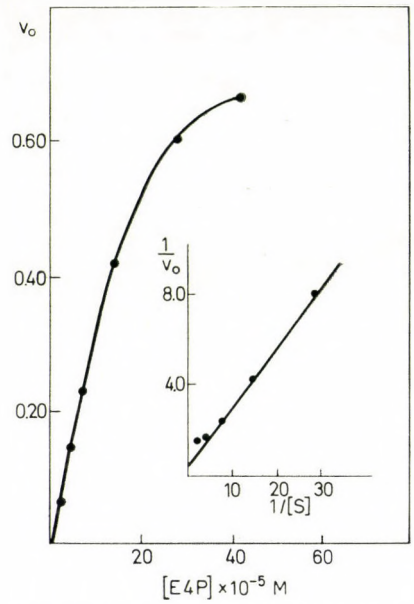


Fig. 3. Effect of erythrose 4-phosphate concentration on the initial velocity of DAHP synthesis. The reaction mixture contained 20 μmoles of Tris-maleate buffer (pH 6.5), 0.45 μmole of phosphoenolpyruvate, 60 μg of protein and erythrose 4-phosphate as indicated in a total volume of 1.0 ml

substrate interaction is sequential and that the product of the first substrate (PEP) dissociates from the enzyme prior to the addition of the second substrate (E4P). On the basis of the kinetic results presented the reaction pathway of enzyme and substrate interaction may be the following

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E' + C$$

$$E' + B \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E'B \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E + D$$

where E and E' are distinct forms of the enzyme, A and B are substrates (in this case PEP and E4P), C and D are products (inorganic phosphate and DAHP). The steady state rate equation for such a mechanism is

$$\frac{E_t}{v_0} = \phi_0 + \frac{\phi_1}{[A]} + \frac{\phi_2}{[B]} \qquad (1)$$

where E_t represents total enzyme concentration; v_0, measured initial velocity; ϕ_0, $1/k_3 + 1/k_7$; ϕ_1, $k_2 + k_3/k_1k_3$; ϕ_2, $k_6 + k_7/k_5k_7$ (Dalziel, 1957). If both substrates were required to be present for the formation of the enzyme substrate

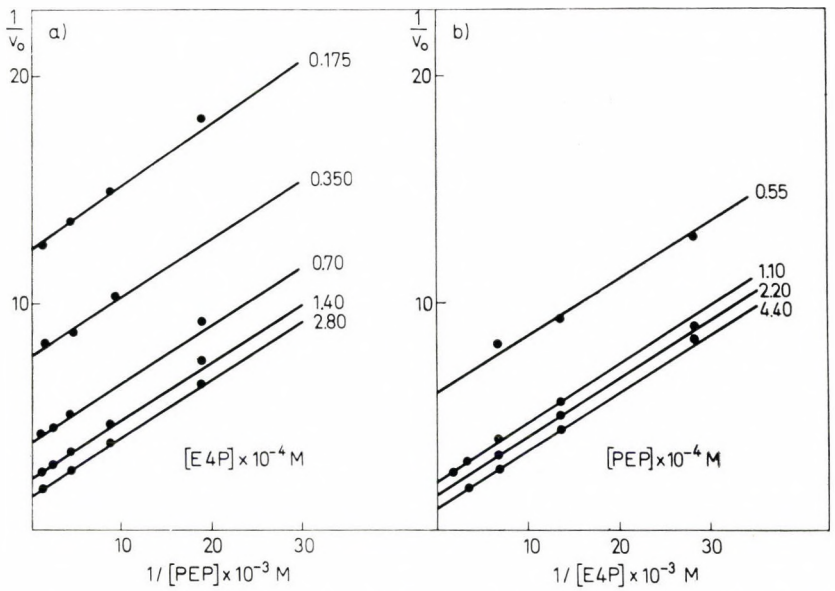


Fig. 4. Double reciprocal plots of the initial rate of DAHP synthesis against phosphoenolpyruvate concentration in the presence of a series of fixed concentrations of erythrose-4-phosphate (a) and against erythrose 4-phosphate concentration in the presence of a series of fixed concentrations of phosphoenolpyruvate (b). The intercepts on the ordinate are the reciprocals of the apparent maximal velocities, $1/V'$, and the intercepts on the abscissa are negative reciprocals of the apparent Michaelis constants, $-1/K_m$. The reaction mixture contained 20 μmoles of Tris-maleate buffer (pH 6.5), 60 μg of protein and the substrates as indicated in a total volume of 1.0 ml

complex, a ternary complex, the steady state rate equation would be

$$\frac{E_t}{v_0} = \phi_0 + \frac{\phi_1}{[A]} + \frac{\phi_2}{[B]} + \frac{\phi_{12}}{[A]\,[B]} \tag{2}$$

Double reciprocal plots of the initial rate data consistent with Eq. 2 should yield a set of linear curves which intersect at a common point. The difference between Eq. 1 and Eq. 2 resides in the absence of the $[A][B]$ term in Eq. 1. If the term ϕ_{12} is very small relative to other terms in Eq. 2, Eq. 1 will be approached. That means that the results presented in Fig. 4 do not exclude by themselves a sequential bireactant mechanism like Ordered BiBi involving the formation of one central ternary complex (Cleland, 1963a).

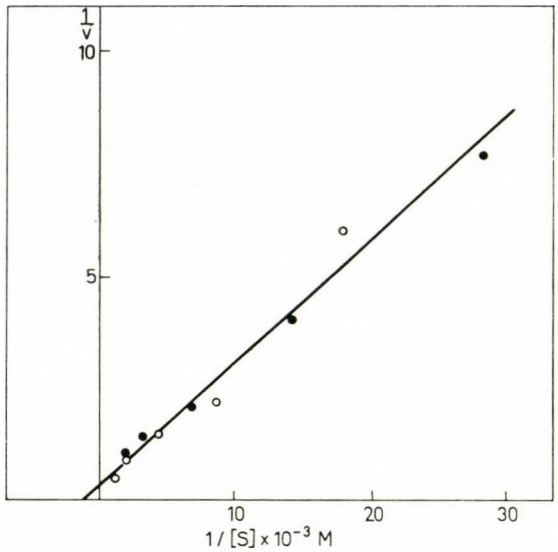


Fig. 5. Secondary plot of the data from Figs 4a and 4b for the determination of the "absolute" Michaelis constant of the enzyme for both substrates. The graphical analysis yields $1/V$ as intercept on the ordinate and $-1/K_{PEP}$, $-1/K_{E4P}$ as intercept on the abscissa. ○——○, $1/V'$ *vs.* 1/[PEP] (from Fig. 4a); ●——●, $1/V'$ *vs.* 1/[E4P] (from Fig. 4b)

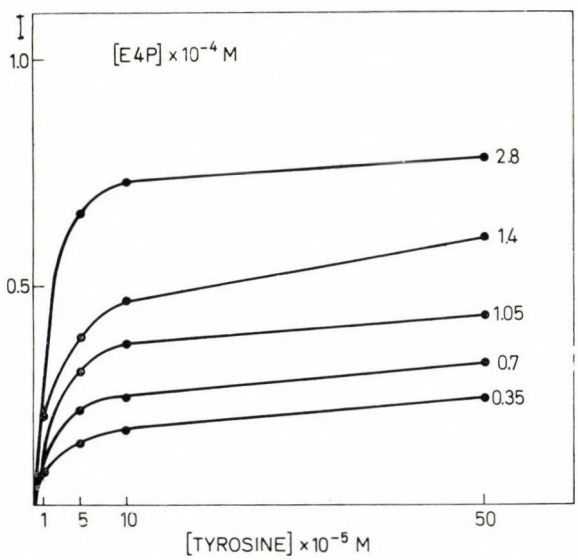


Fig. 6. Effect of erythrose 4-phosphate concentration on the inhibitory effect of tyrosine. The reaction mixture contained 20 μmoles of Tris-maleate buffer (pH 6.5), 0.45 μmole of phosphoenolpyruvate, 140 μg of protein, erythrose 4-phosphate and tyrosine as indicated in a final volume of 1.0 ml

Inhibition of the activity of the enzyme with tyrosine

Smith et al. (1962) found that the inhibition of the activity of the enzyme is non-competitive for both substrates. During our studies we have observed that in the presence of a fixed concentration of PEP, the inhibitory effect of tyrosine

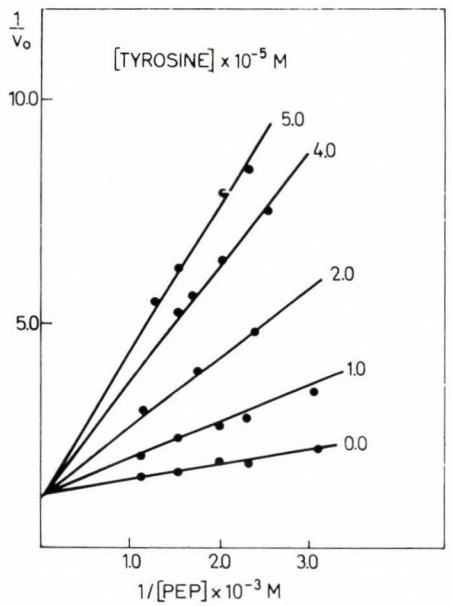
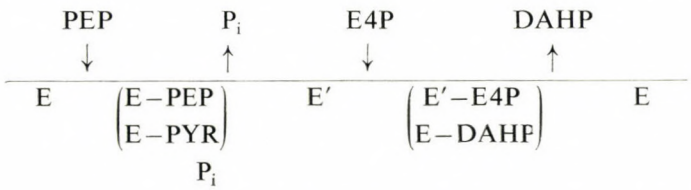


Fig. 7. Linear competitive inhibition of DAHP formation for phosphoenolpyruva te by tyro sine. Double reciprocal plot of the initial rates of DAHP formation *vs* phosphoenolpyruvate concentration in the presence of a series of fixed concentrations of tyrosine and fixed concentration of erythrose 4-phosphate. The reaction mixture contained 20 μmoles of Tris-maleate buffer (pH 6.5), 0.22 μmole of erythrose 4-phosphate, 140 μg of protein, phosphoenolpyruvate and tyrosine as indicated in a total volume of 1.0 ml

depends on the concentration of E4P. As shown in Fig. 6 the inhibitory effect of tyrosine increases in the presence of increasing concentrations of E4P. Supposing on the basis of kinetic evidences presented in this paper, that the mechanism of action of the enzyme is Ping-Pong, the explanation of the results may be the following:



In the presence of PEP and in the absence of E4P the enzyme is transformed to a stable intermediate E' containing covalently bound pyruvyl group. This derivative of the enzyme does not form an enzyme-inhibitor complex in the presence of tyrosine. In the presence of E4P, as the concentration of the compound increases, the probability of the formation of free enzyme E also increases, because

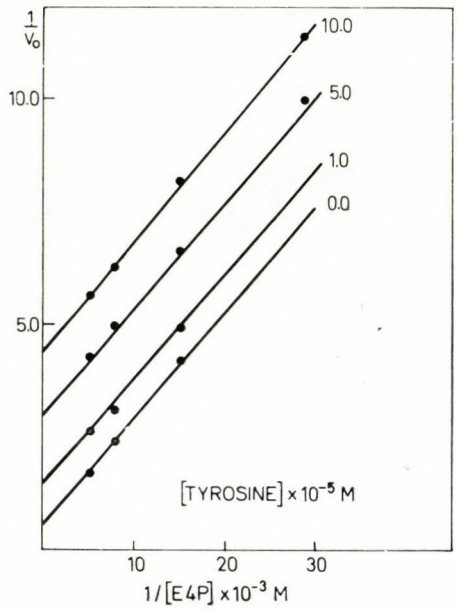


Fig. 8. Linear uncompetitive inhibition of DAHP synthesis for erythrose 4-phosphate by tyrosine. Double reciprocal plot of the initial rate of DAHP formation *vs* erythrose 4-phosphate concentration in the presence of different fixed concentrations of tyrosine and fixed concentration of phosphoenolpyruvate. The reaction mixture contained 20 μmoles of Tris-maleate buffer (pH 6.5), 0.44 μmole of phosphoenolpyruvate, 140 μg of enzyme, erythrose 4-phosphate and tyrosine as indicated in a total volume of 1.0 ml

the catalytic pathway is completed, and in the presence of tyrosine the inactive *EI* complex is formed. This explanation involves the conclusion that since only the free enzyme combines with tyrosine to form the inactive *EI* complex, the inhibition of the activity of the enzyme by tyrosine must be competitive for PEP in contrast to previous observations (Smith et al., 1962). Because of the discrepancies between previous observations and our results we have analyzed in detail the mechanism of inhibition by tyrosine of the activity of the enzyme. The initial rate equation for the forward reaction in the absence of inhibitor is:

$$v = \frac{V_1 [\mathrm{PEP}] [\mathrm{E4P}]}{K_{\mathrm{E4P}} \mathrm{PEP} + K_{\mathrm{PEP}} \mathrm{E4P} + \mathrm{PEP} + \mathrm{E4P}} \qquad (3)$$

and in the presence of inhibitor when the inhibitor combines only with the free enzyme E to form EI complex it is:

$$v = \frac{V_1 [\text{PEP}] [\text{E4P}]}{K_{\text{E4P}} [\text{PEP}] + K_{\text{PEP}} \left(1 + \frac{\text{TYR}}{K_i}\right) [\text{E4P}] + [\text{PEP}] [\text{E4P}]} \tag{4}$$

When PEP is the variable substrate and the concentration of E4P is constant, the inverted Eq. 4 will be

$$\frac{1}{v} = \frac{K_{\text{PEP}}}{V_1} \left(1 + \frac{[\text{TYR}]}{K_i}\right) \left(\frac{1}{[\text{PEP}]}\right) + \frac{1}{V_1} \left(1 + \frac{K_{\text{E4P}}}{[\text{E4P}]}\right) \tag{5}$$

and according to Eq. 5 the inhibition is competitive. The plot of the reciprocal of the initial rate of production of DAHP against the reciprocal of PEP concentration in the absence and presence of various concentrations of tyrosine gives a set of lines intersecting on the ordinate as shown in Fig. 7. The inhibition of the activity of the enzyme by tyrosine is competitive for PEP, as predictable from Eq. 5. When E4P is the variable substrate and the concentration of PEP is constant, the inverted Eq. 4 will be

$$\frac{1}{v} = \frac{K_{\text{E4P}}}{V_1} \left(\frac{1}{\text{E4P}}\right) + \frac{1}{V_1} \left(1 + \frac{K_{\text{PEP}}}{[\text{PEP}]}\right) \left(1 + \frac{[\text{TYR}]}{K_i 1 + \frac{[\text{PEP}]}{K_{\text{PEP}}}}\right) \tag{6}$$

and according to Eq. 6 the inhibition is uncompetitive. The double reciprocal plots of the initial rate of synthesis of DAHP *vs* E4P concentration in the absence and presence of various concentrations of tyrosine gives a set of parallel lines as shown in Fig. 8. The inhibition of the activity of the enzyme by tyrosine is uncompetitive for E4P.

A secondary plot of the slopes *vs* tyrosine concentrations from Fig. 7 gives a straight line as shown in Fig. 9 and the $K_{i\ \text{slope}}$ for the competitive inhibition is 4.5×10^{-6} M. Secondary plot of the intercepts *vs* tyrosine concentrations from Fig. 8 (Fig. 10) gives also a straight line and the $K_{i\ \text{intercept}}$ for the uncompetitive inhibition is 2.0×10^{-5} M. Because of the mechanism of enzyme action and of inhibition, $K_{i\ \text{slope}}$ may be equal to K_i according to Eq. 5.

From this type of inhibition experiments it is possible to calculate the absolute Michaelis constants of the enzyme for both substrates (Cleland, 1963b). In the experiments presented in Fig. 8 the fixed concentration of PEP is 4.4×10^{-4} M and since $K_{i\ \text{intercept}}$ from Eq. 6 is $K_i(1 + [\text{PEP}]/K_{\text{PEP}})$ the value of K_{PEP} is 1.0×10^{-3} M. In the experiment presented in Fig. 7 when PEP is the variable substrate the slope of the curve in the absence of inhibitor from Eq. 5 is K_{PEP}/V_1, equal to 0.26×10^{-3} and $1/V_1$ is 0.24. The intercept of this line from Eq. 5 is $(1/V_1)(1 + K_{\text{E4P}}/[\text{E4P}])$ and its numerical value is 2. Since the fixed concentration

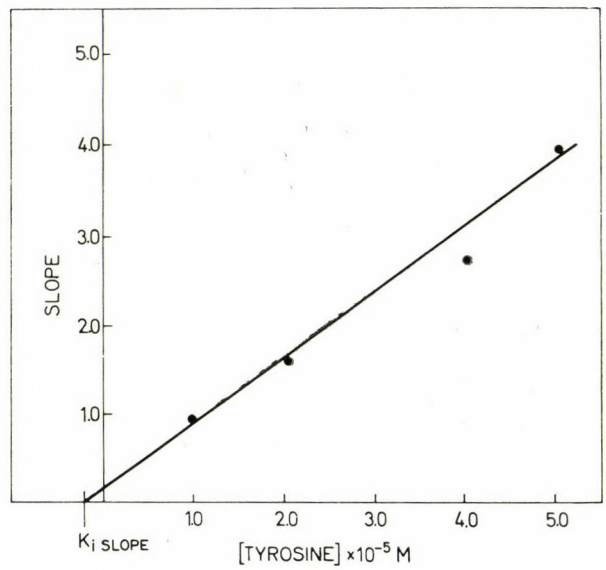


Fig. 9. Secondary plot of the slopes of linear curves *vs* tyrosine concentration from Fig. 7 for the determination of $K_{i\,\text{slope}}$. The straight line gives $K_{i\,\text{slope}}$ as an intercept on the abscissa

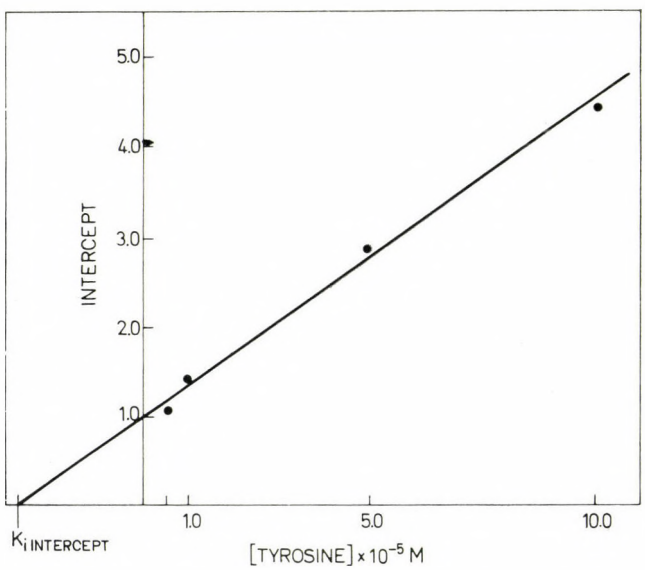


Fig. 10. Secondary plot of the ordinate intercepts *vs* tyrosine concentration from Fig. 8 for the determination of $K_{i\,\text{intercept}}$. The straight line gives $K_{i\,\text{intercept}}$ as an intercept on the ordinate

of E4P in the experiment (Fig. 7) is 2.2×10^{-4} M, K_{E4P} is 1.0×10^{-3}. The absolute Michaelis constants of the enzyme for PEP and E4P calculated from the inhibition experiments are the same, as the Michaelis constant obtained from independent experiments presented in Figs 4 and 5.

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Structural Investigations on Pancreatic α-Amylase

I. Difference Spectra and Optical Rotatory Dispersion Measurements

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1. Optical rotatory dispersion, denaturation difference spectra and perturbation difference spectra of native and denatured hog pancreas α-amylase(α-1,4-glucan 4-glucanohydrolase, E.C. 3.2.1.1) were studied.

2. From the $-b_0$ and $[m']_{232}$ rotatory parameters a helix content of about 15 per cent can be calculated.

3. Spectrophotometric titration of phenolic ionization is anomalous and irreversible with pK-s of ionization 11.5 and 10.95 in the forward and reverse direction, respectively. Removal of bound calcium decreases the pK to 10.9 in the forward titration.

4. Low pH and sodium dodecyl sulfate cause only a partial exposure of buried chromophores to the solvent medium as it can be demonstrated by the difference spectrum measurements.

5. In native state 80 per cent of tryptophans and only 30 per cent of tyrosines are accessible to 20 per cent ethylene glycol as it was measured by the perturbation technique. In 92 per cent D_2O all tryptophans become exposed while the amount of accessible tyrosines remain unchanged (70 per cent buried).

α-Amylase, beside proteases and ribonuclease, is a pancreatic enzyme which is involved in the decomposition of high molecular weight substrates. While the other pancreatic enzymes were extensively studied and detailed data are known about the connection between their structure and function, limited amount of information about amylase is available in this respect. The essential role of calcium in the activity as well as in the structural stability of amylase has been known for a long time (Fischer et al., 1958). Calcium also protects the enzyme against urea inactivation (Hatfaludi et al., 1966). It has also been shown that crystalline amylase can be resolved by ion exchange chromatography into two fractions of the same specific activity (Szabó, Straub, 1966; Juhász, Szabó, 1967).

In this paper investigations are presented on the conformation of the polypeptide chain and on the distribution of aromatic chromophores in α-amylase. The general experience is (Laskowski, 1966) that both tryptophan and tyrosine are roughly equally distributed between the polar and non-polar parts of the proteins studied until now. Pancreatic α-amylase, however, differs from other proteins

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because all its tryptophyl side chains are practically in accessible position for water (D_2O) while about 70 per cent of the tyrosines are deeply buried in native state.

Experimental Part

Materials

Hog pancreas α-amylase was prepared according to Hatfaludi et al. (1966) and was kindly supplied by Dr M. T. Szabó. The protein was twice recrystallized and stored as a crystal suspension in 0.01 M tris buffer pH 7.4 containing 0.001 M calcium chloride. The specific activity of preparations varied between 4000 and 5000 amylase units (Smith, Roe, 1949).

Protein concentration was determined spectrophotometrically in 0.01 M tris buffer pH 7.4 at 280 mμ using the extinction coefficient of $E_{1cm}^{1\%} = 24$. All calculations were based on a molecular weight of 50 000 determined by Dr P. Závodszky, in sedimentation equilibrium experiments (private communication).

For the removal of calcium EDTA was added to a final concentration of 0.01 M in 0.01 M tris buffer pH 7.4 to a solution containing 2 to 3 mg protein per ml. The solution was dialyzed against several changes of 0.01 M EDTA for 70 hours at 5°C. It was supposed that this treatment was satisfactory to remove practically all the firmly bound calcium.

N-acetyl-tryptophan ethyl ester, N-acetyl-tyrosine ethyl ester and ethylene glycol were products of Reanal (Budapest). D_2O (99.5%) was supplied by the Isotope Institute (Budapest). Polyethylene glycol (Carbowax 400) was purchased from Carlo Erba Co. (Italy).

Urea was recrystallized twice from 70 per cent ethanol and a fresh solution was prepared daily. Sodium dodecyl sulfate was a product of DuPont-Nemours Co. and was twice recrystallized from ethanol. All other substances used were of reagent grade.

Methods

Absorption was measured with Spectromom 201 spectrophotometer. Difference spectra were recorded between 260 and 310 mμ with Unicam SP 700 recording spectrophotometer in 1 cm silica cells. The protein concentration in these measurements varied between 0.6 and 0.8 mg per ml, which corresponds to 1.4 – 1.9 optical density and a slit width less than 0.05 mm at 280 mμ.

In the spectrophotometric titrations the pH of the solutions was adjusted with 0.25 M glycine-sodium hydroxyde buffer. Samples were incubated for about one hour. pH was controlled before and after the spectrophotometric assay with a Radelkisz OP 205 type precision pH meter of an accuracy of ±0.02 to 0.05 pH in the alkaline range.

Solvent perturbation measurements were carried out between 260 and 310 mμ in double-sector cells as described by Herskovits and Laskowski (1960, 1961). 20 per cent ethylene glycol and polyethylene glycol, as well as 92 per cent D_2O were used as perturbing medium. Stray light was checked according to Mihályi (1965). The maximum optical density of the reference solutions varied between 1.8 and 2.4. Under these conditions stray light can be neglected.

In the optical rotatory experiments a Polarmatic 62 type recording spectropolarimeter (Bellingham and Stanley, London) was used. The rotatory constants were calculated from the tracings between 350 and 580 mμ according to the equation of Moffitt (1956) as follows:

$$[m'] = \left(\frac{MRW}{100} \frac{3}{n^2+2}\right)[\alpha]_\lambda = \frac{a_0 \lambda_0^2}{(\lambda^2 - \lambda_0^2)} + \frac{b_0 \lambda^4}{(\lambda^2 - \lambda_0^2)^2}$$

where λ_0 was taken as 212 mμ. For the MRW mean residue weight 115 was calculated from the amino acid composition (Dr T. Dévényi, private communication). For refractive index corrections the data listed in Fasman's paper (Fasman, 1963) were applied. The measurements were carried out in 1 cm cells mounted with silica windows. The protein concentrations varied between 1.5 to 3.0 mg per ml in the 350–580 mμ region and 0.05 to 0.08 mg per ml in the range of anomalous dispersion (215 to 250 mμ). The signal to noise ratios were 20 and 14 in the above two wavelength ranges, respectively. All measurements were carried out at room temperature.

Reduced amylase was prepared in the following way: protein was dissolved to 10–15 mg per ml concentration in 8 M urea containing 0.01 M EDTA and 0.01 M tris buffer pH 8.5. To the solution mercaptoethanol was added to 0.3 M final concentration and was incubated for 16 hours. Then iodoacetate was added in a 3 fold molar excess to mercaptoethanol and the solution was incubated for 30 minutes. Protein was precipitated and washed 3 times with acidic acetone (acetone: 1 M hydrochloric acid in 20 : 1 ratio v/v). The precipitate was readily soluble in 0.01 M tris buffer, pH 8.4. This solution was dialyzed against tris buffer, pH 8.0 and was filtered.

Results and Discussion

Optical rotatory dispersion of amylase

The optical rotatory dispersion of amylase was investigated in the 215 to 580 mμ wavelength range under different conditions. Between 350 and 580 mμ the dispersion of native and denatured amylase is simple and the Moffitt constants calculated from the dispersion curves are listed in Table 1. In the 270–290 mμ range a shoulder can be observed in the native amylase in 0.01 M tris buffer pH 7.4, which does not disappear completely upon denaturation with 8 M urea of acidic pH.

Table 1

Optical rotatory data of α-amylase

Experimental conditions	$-a_0$	$-b_0$	$-[m']_{232}$
In 0,01 M tris, pH 7.4	260	115	2800
In 0.01 M HCl, pH 2.5	235	55	2100
In 8 M urea	400	55	2150
In 8 M urea + 0.05 M β-mercaptoethanol	455	55	2800
In sodium dodecyl sulfate (2 : 1 w/w to protein)	310	120	4250

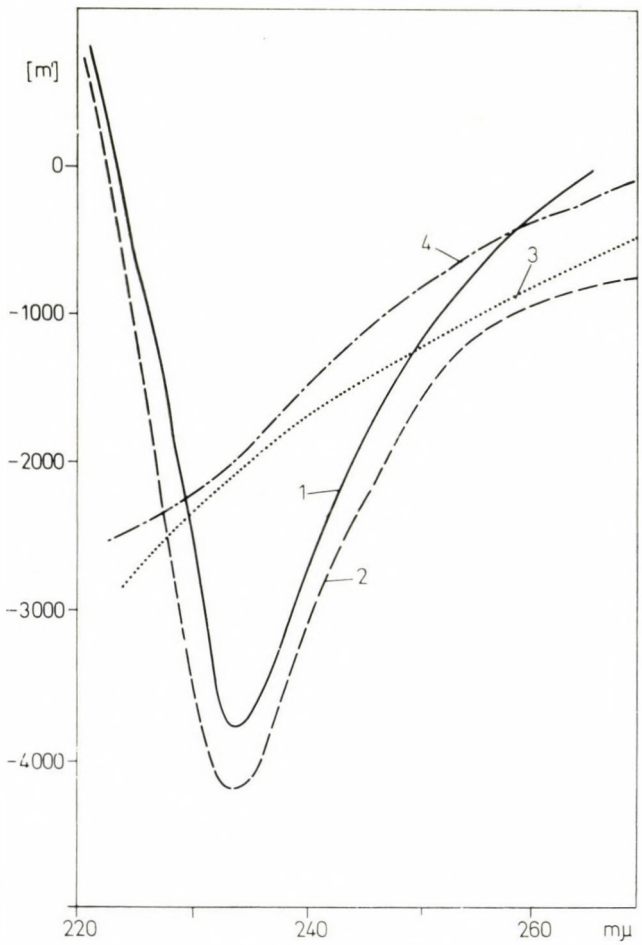

Fig. 1. Far ultraviolet Cotton effect of α-amylase. 1 — in 0.01 M tris buffer, pH 7.4; 2 — in the presence of sodium dodecyl sulfate (2 : 1 w/w to protein); 3 — in 8 M urea, pH 7.5; 4 — in 0.02 M hydrochloric acid, pH 2

A pronounced Cotton effect can be detected at the shorter wavelengths with a trough at 232 mμ (Fig. 1). The mean residue rotation [m'] calculated from the trough of native amylase can also be seen in Table 1. As it can be also seen in Fig. 1 both acidic pH and concentrated urea change the anomalous dispersion to a simple one, while the presence of sodium dodecyl sulfate slightly increases the negativity of the Cotton effect. Both the b_0 Moffitt constant, which is in a fair agreement with the value determined by Jirgensons (1965), and $[m']_{232}$, the mean residue rotation, reflect a low periodic organization in the polypeptide chain of α-amylase. The right handed α-helix content does not exceed 15 per cent, as it can be calculated from the values shown in Table 1. It can be presumed that amylase like other pancreatic enzymes, such as chymotrypsin and ribonuclease, contains β-structure as periodic arrangement.

Spectrophotometric titration

The ionization of phenolic groups was studied by measuring the changes in absorption at 295 mμ both from neutral to alkaline direction (forward titration) and from pH 13 to the neutral direction (reverse titration). The results are shown in Fig. 2.

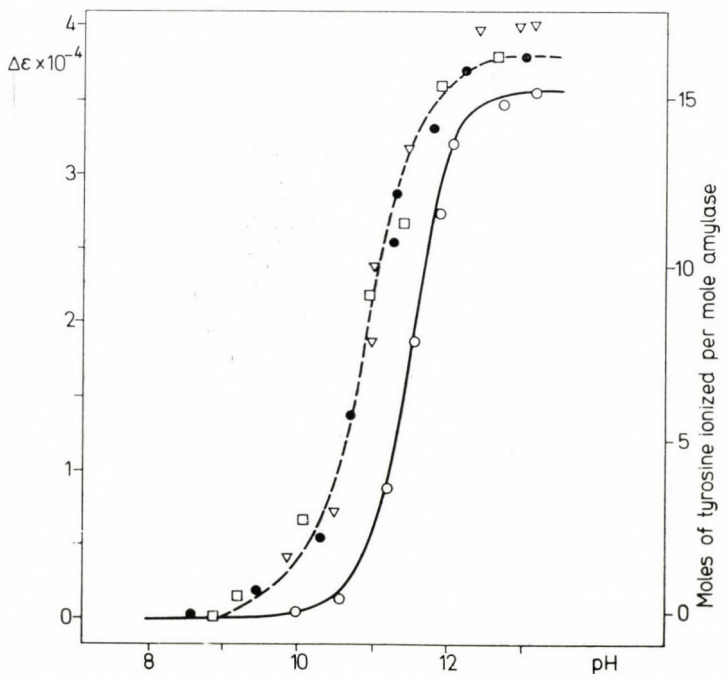

Fig. 2. Spectrophotometric titration of α-amylase in 0.25 M glycine-sodium hydroxyde buffer. Open symbols — forward titration, solid symbols — reverse titration, ○ — native amylase, ▽ — amylase in 8 M urea, □ — "Ca-free" amylase

It can be seen in Fig. 2, that the dissociation of phenolic hydroxyl groups in native amylase is anomalous. It begins in the forward direction only above pH 10 and has a pK of 11.4. The titration is not reversible and the curve obtained in the forward direction differs from that in the reverse direction. The pK of reverse titration is 10.95. The same pK was observed when titration was carried out in 8 M urea.

15.5 moles of tyrosine per mole of amylase were found to be in ionized state on the basis of the absorption difference measured at pH 13.0 after 30 minutes of incubation. A complete ionization of all 16 tyrosine groups can be achieved only after at least one hour incubation.

The titration of "Ca-free" amylase in the forward direction is similar to that of the Ca-containing protein in the reverse direction having a pK of 10.9 (Fig. 2). This indicates that upon removal of bound calcium alterations occur in the structure of the enzyme which normalize the ionization of tyrosine.

Both the irreversibility of titration and the high pK of ionization suggest that native amylase contains buried tyrosyl side chain. It can also be seen that the protein is fairly resistant to denaturation with alkali. The data obtained are in agreement with the findings of Fischer et al. (1958) concerning the stabilizing effect of bound calcium.

Difference spectra

From the spectrophotometric titration it is evident that the sizeable amount of aromatic chromophores of α-amylase is in buried condition. This is also supported by the fact that pancreatic α-amylase is a quite "hydrophobic" protein containing a relatively low amount of polar side chains. Amylase contains 10 tryptophyl and 16 tyrosyl residues per 50 000 molecular weight (Dr T. Dévényi, private communication). Difference spectra were supposed to supply further informations on the localization of chromophoric side chains.

The difference spectra were studied under different denaturing conditions, i.e. at pH 2.5, in the presence of sodium dodecyl sulfate (2 : 1 weight ratio to protein) and in 8 M urea. Spectra were recorded against amylase in 0.01 M tris buffer pH 7.4.

It can be seen in Fig. 3 that different denaturing agents cause denaturation to different extents as far as changes in absorption are concerned. The most pronounced effect was observed in the presence of urea after four hours incubation before recording the spectrum.

Both acidic pH and sodium dodecyl sulfate cause a smaller change in the exposure of tyrosine and tryptophan than urea. Moreover, the absorption changes are greater at the shorter wavelength maximum (287 mμ) than at the longer one (293 mμ). The difference maximum at 287 mμ is connected mainly with tyrosine, therefore under these conditions tyrosine is involved in the changes of absorption. Tryptophan (at 293 mμ) changes to a lesser extent upon acidification to pH 2.5 or upon addition of sodium dodecyl sulfate. This can also be seen when the

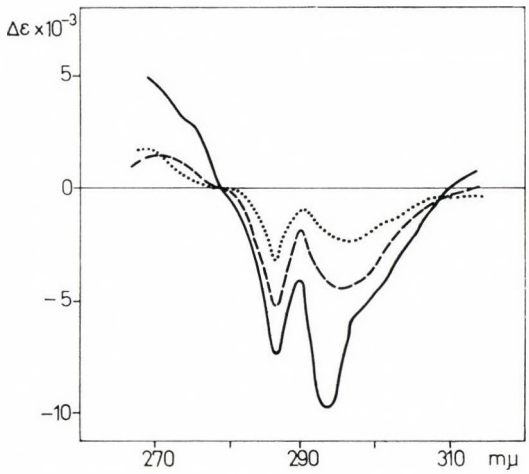

Fig. 3. Denaturation difference spectra of α-amylase. Reference solution was amylase in 0.01 M tris buffer pH 7.4, ——— in 8 M urea, ... in hydrochloric acid pH 2.5, - - - - - in the presence of sodium dodecyl sulfate (2 : 1 w/w ratio to protein)

molar absorption changes at 287 mμ and 293 mμ under different conditions are compared, as follows: in 8 M urea 7100 and 9600, in the presence of sodium dodecyl sulfate 5400 and 5300, at acidic pH values 3500 and 2500, respectively. The incomplete exposure of chromophores in the presence of sodium dodecyl sulfate and at acidic pH suggests that the molecule is relatively insensitive to these denaturing effects.

Perturbation difference spectra

The distribution of chromophores in α-amylase was investigated by perturbation technique (Herskovits, Laskowski, 1960, 1961). The perturbation difference spectra of amylase were investigated in tris buffer pH 7.4, and in 7 M urea in the presence of 20 per cent ethylene glycol. A mixture of model amino acids i.e. N-acetyl-tyrosine ethyl ester and N-acetyl tryptophan ethyl ester in different molar ratios was also studied.

Calculations were based on molar perturbation difference values of $\Delta\varepsilon_{285} = 100$ for tyrosine and $\Delta\varepsilon_{291} = 250$ for tryptophan (Móra, Elődi, 1968).

A partial exposure of tyrosine and tryptophan was observed only when the perturbation difference spectrum was recorded in the presence of 7 M urea after 3–4 hours incubation (Fig. 4b, curve 6). A complete accessibility, however, of all these chromophores was found when amylase was incubated first with concentrated urea (actual urea concentration about 9 M), then ethylene glycol was added, which decreased the urea concentration to 7 M and the spectrum was recorded immediately. This spectrum (Fig. 4b, curve 5) coincided fairly well with

that of the tyrosine-tryptophan model mixture corresponding to their total amount in the protein (Fig. 4b, curve 4).

The spectrum obtained in the above manner showed a gradual change and a decrease of the height of both maxima was observed when the tracing was repeated subsequently. After about four hours incubation, no more changes were

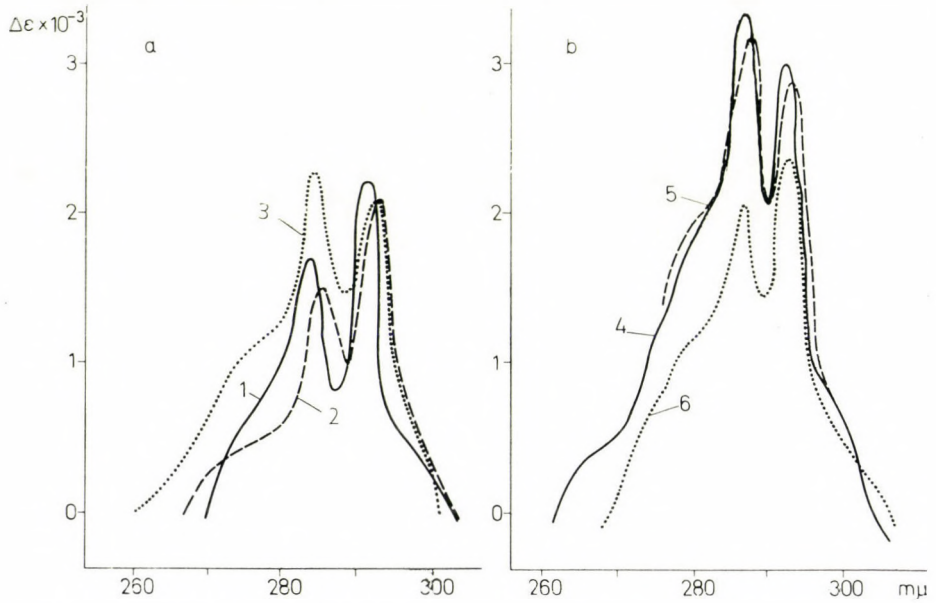

Fig. 4. Solvent perturbation difference spectra of α-amylase in 20 per cent ethylene glycol. a — in 0.01 M tris buffer pH 7.4, 1 — tryptophan-tyrosine mixture in a molar ratio of 8 : 5, 2 — native amylase, 3 — "reduced" amylase (see Methods); b — in 7 M urea, 4 — tryptophan-tyrosine mixture in a molar ratio of 10 : 16, 5 — amylase, recorded immediately after mixing urea treated protein with ethylene glycol, 6 — the same after four hours incubation

observed and a curve similar to that seen in Fig. 4b, curve 6 was obtained. This phenomenon is connected with the reversibility of the effect of urea on amylase. This will be reported in a separate paper (Krysteva, in preparation).

In native amylase 8 tryptophans, out of 10 and 5 tyrosines, out of 16, are in accessible position for ethylene glycol (Fig. 4a, curve 2). The perturbation spectrum of a model mixture of the above composition coincided fairly well with that of the protein (Fig. 4a, curve 1).

The reduction of four disulfide bridges causes an increase in the perturbation of tyrosines only. In reduced amylase 8 tryptophans and 13 tyrosines were found to be accessible for ethylene glycol (Fig. 4a, curve 3). This means that reduction of disulfide bridges and simultaneous removal of bound calcium (see Methods) does not influence the accessibility of the two buried tryptophans while that of

tyrosines was profoundly changed, because 8 additional tyrosines became exposed to the external solvent environment.

The effect of D_2O and polyethylene glycol (degree of polymerisation 400) was also studied. The number of residues exposed to these perturbants are listed in Table 2. It can be seen that the exposure of tyrosines in native amylase does

Table 2

Accessible chromophoric groups of native α-amylase for different perturbing agents in 0.01 M tris buffer, pH 7.4

Perturbant	Number of residues per molecular weight of 50 000	
	tryptophan	tyrosine
D_2O, 92 per cent	10	5
Ethylene glycol, 20 per cent	8	4—5
Polyethylene glycol, 20 per cent	6	4

not depend on the perturbing agent. All buried tyrosines are shielded from D_2O just as well as from polyethylene glycol. This means that the buried tyrosines are deeply imbedded in the interior of the molecule and even water cannot penetrate to them. On the other hand, all tryptophans are accessible for D_2O and only four are inaccessible for polyethylene glycol.

From the data presented pancreatic α-amylase appears to be a molecule of low periodic organization. In contrast to other proteins investigated until now in pancreatic α-amylase all ten tryptophyl side chains are accessible for water, i.e. they are located on the surface or near the surface. A greater part of tyrosyl side chains, however, are more or less deeply imbedded in the interior part of the molecule and are exposed to the solvent environment when the disulfide bonds are reduced.

The investigation of other structural properties of this protein is in progress in our laboratory.

Acknowledgement

The authors are very much indebted to Dr M. T. Szabó for the generous supply of crystalline α-amylase preparations. The discussions with Dr F. B. Straub in the course of the investigations were most helpful.

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A Thermostable Factor Prepared from Rat Liver Inhibiting Protein Synthesis

Short Communication

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It has been observed that a thermostable component of rat liver homogenate inhibits the incorporation of radioactive amino acids in a rat liver ribosomal system (Fig. 1). In order to characterize the nature of this inhibition, we have purified to some extent this inhibitory principle and studied its effect on valyl-t-RNA-synthetase.

The inhibitory factor – Factor I – was prepared from rat liver. 50 g of liver tissue were homogenized in two volumes of 0.1 M Tris-HCl buffer, pH 7.4, and centrifuged at 105 000 *g*. The supernatant fluid was heated at 90°C for 6 minutes, the precipitated material was removed by centrifugation. The solution was treated with charcoal, 40 mg per ml. The material not adsorbed to charcoal was precipitated by the addition of ethanol to a concentration of 66% (v/v). This precipitate was discarded and the ethanol solution was evaporated and taken up in 100 ml of Tris-HCl buffer, pH 7.4. 0.05 ml of this solution – Factor I-a – used in the experiments mentioned below is therefore equivalent to 25 mg fresh liver.

Valyl-t-RNA-synthetase was assayed by measuring the rate of the charging of valyl-t-RNA in the following reaction mixture. 0.35 mg of rat liver t-RNA, 0.5 μmole of adenosinetriphosphate, 1.0 μmole of Mg-acetate, 50 μmoles of KCl, 3 μmoles of mercaptoethanol, 50 μmoles of Tris-HCl buffer, pH 7.4, and 0.5 mg of a rat liver 105 000 *g* supernatant protein were incubated with 1 μCi of ^{14}C–valine, uniformly labeled 91 mCi per mole, at 37°C for 10 minutes in a total volume of 0.5 ml. The reaction mixture was arrested by precipitating the t-RNA with 3 ml of 10% cold trichloroacetic acid. The precipitate was washed three times at 0°C with trichloroacetic acid and once with 3 ml of 96% cold ethanol. The acid insoluble residue was collected and radioactivity was determined in a Friesecke–Hoepfner gas flow-counter.

t-RNA was isolated from rat-liver with a slight modification of Kirby's method (Kirby, 1956) and was further purified by chromatography on DEAE-cellulose according to Holley (1961).

Factor I-a turned out to be dialysable. Further the inhibitory effect vanished after combustion. Therefore, it is assumed that the factor is an organic compound of low molecular weight. In all probability it is not a nucleotide, as it is not bound

to charcoal and has no absorbancy at 260 mμ. Amino acid analysis has shown that Factor I-a is not contaminated by valine in such quantities as to depress the specific activity of the radioactive valine used.

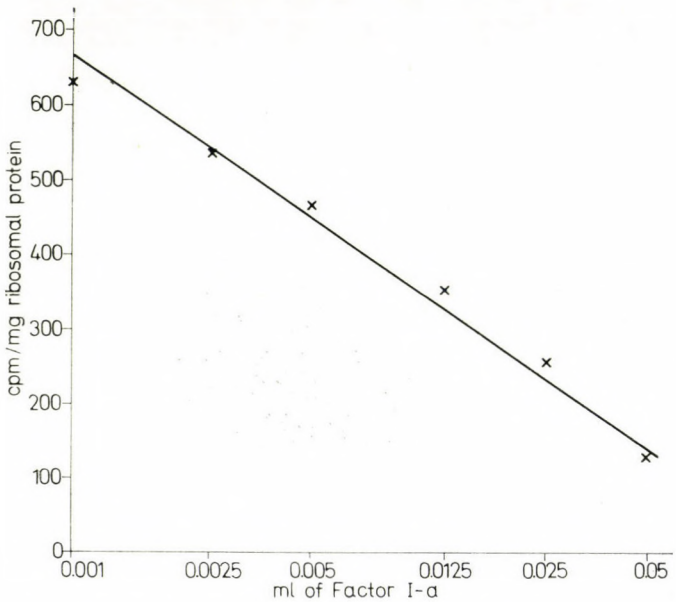

Fig. 1. Inhibition of amino acid incorporation in a rat liver ribosomal system. Incorporation of radioactive amino acid in rat liver ribosomal system was assayed in the following reaction mixture: 0.5 mg ribosomal protein, ATP, 0.5 μmole; GTP, 0.1 μmole; Mg-acetate, 5.0 μmoles; NH_4Cl, 12.5 μmoles; KCl, 12.5 μmoles; mercaptoethanol, 3.0 μmoles; Tris-HCl, pH 7.8, 50 μmoles and 0.5 mg of rat liver 105 000 *g* supernatant protein (as a source of synthetase and "transfer-enzymes"). Samples of a final volume of 0.5 ml were incubated for 20 min in the presence of 1 μCi of ^{14}C valine. The incubation was stopped by precipitating the proteins with 10% trichloroacetic acid and the samples were prepared for counting roughly according to the method of Rabinowitz et al. (1954)

Table 1

Inhibiting activity of Factor I-a on the charging of t-RNA

Conditions	Cpm per reaction mixture in isolated *t*-RNA
Total mixture*	2607
Total mixture + 0.05 ml Factor I-a**	915
Total mixture after heating at 90°C for 15 min	70

* see the text
** see the text

The inhibitory action of Factor I-a on the amino acid incorporation in a rat liver ribosomal system seems to be in connection with the inhibition of amino acid synthetases. As shown in Table 1 the charging of t-RNA is inhibited by Factor I-a by about 60%. It was found that the inhibitory effect of this factor does not change if the fresh rat liver homogenate is incubated for 30 minutes at 37°C prior to the preparation of Factor I.

Though the biological role of this factor is not yet elucidated, it is supposed that it might play some part in the regulation of protein synthesis.

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Studies on the Shape Regeneration of Preserved Erythrocytes

Short Communication

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(Received July 30, 1968)

According to Jacob (1966) disk-sphere transformation of erythrocytes is due to the so called membrane fragmentation. This appears in the form of a symmetrical lipid release which would cause sphering by decreasing cell surface. Weed and Reed (1966) present the same phenomenon as the cause of microsphaerocytosis developing in the course of blood preservation. On the other hand, Nakao and coworkers (1960) have demonstrated that regenerating the ATP* content of erythrocytes preserved for 6—8 weeks by adenine and inosine the cells regain their disk shape. If we accept membrane fragmentation as a cause of microsphaerocytosis, this shape regeneration would be conceivable only in that case, when the cell after having restored its energy supply, would increase its surface again by replacing the released membrane constituents. An increase of surface by cholesterol uptake with a concomittant discoid transformation has already been observed in connection with inhibited serum cholesterol esterase activity (Cooper, Jandl, 1966).

Weed and Reed (1966) have given an account of the conditions which permit the separation of the fragments from erythrocytes in the centrifuge. Keeping to these conditions microsphaerocytaer erythrocytes preserved in ACD for 6 weeks were washed six times with four volumes of cold isotonic sodium chloride containing 2.5 mM magnesium chloride and 15 mM inorganic phosphate, pH 7.4. The supernatant fluid containing the fragments was thoroughly sucked off. ATP level was regenerated at 37 °C by adding 10 mM inosine and 1 mM adenine. The transitory and stable microscopic forms of the disk-sphere transformation were characterized by arbitrary numbers from 1.0—0.0. The percentage distribution values were multiplied by these factors and the results were summarized and named morphological index (I_m) (Gárdos et al., 1966).

In our preliminary experiments it was found that the discoid transformation of washed erythrocytes suspended in crystalloid medium was slower and smaller than that of washed erythrocytes suspended in their plasma containing the fragments. It turned out, however, that compatible, fresh ACD plasma, and even albumin, isolated by gel-filtration had the same advantageous effect.

* Abbreviations: ATP = adenosine 5'-triphosphate; ACD = acid citrate dextrose.

At the same time gamma globulin and macroglobulin isolated by gel-filtration as well as lecithin, cholesterol and oleic acid applied alone or together with these protein fractions within wide concentration ranges did not influence shape regeneration in an advantageous way or directly inhibited it. The sphering effect

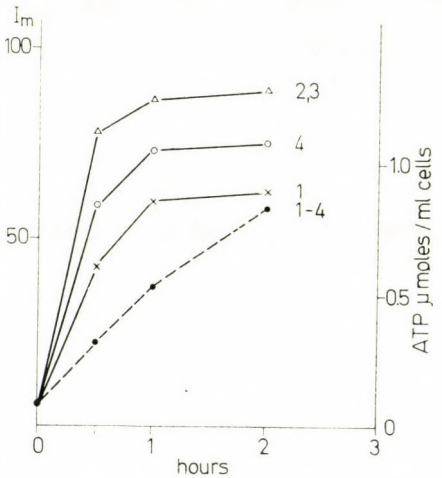

Fig. 1. Effect of 1 mM adenine and 10 mM inosine on the ATP content and shape of erythrocytes preserved for 6 weeks. The tubes contained 5 ml of washed erythrocyte sediment and 5 ml of different media

Media: 1: 160 mM NaCl; 2: normal serum; 3: 4.9 mg/ml albumin in 160 mM NaCl; 4: 0.49 mg/ml albumin in 160 mM NaCl

The broken line indicates ATP concentration, the solid lines mark I_m, the "morphological index" (Gárdos et al., 1966)

of lecithin (Jandl, Tomlinson, 1958) and oleic acid (Braasch, 1968) has already been known from the literature. Fig. 1 represents the results of a typical experiment. While ATP level is regenerated independently of the protein content of the suspending medium, for maximum morphological regeneration the presence of albumin or its subsequent addition is necessary. Albumin exerts an advantageous effect even in one tenth of the physiological concentration, but this effect is not complete. These findings of ours revive once more the discoveries of Furchgott and Ponder (1940) in connection with the antisphering effect of albumin.

To interpret the partial morphological regeneration of the washed cell we investigated whether it still had albumin bound to its surface. As it is known from the works of Müller and Gramlich (1963) the relative amount of albumin in the protein film of the erythrocyte surface decreases in the course of washings, and according to Árky and coworkers (1968) after three washings no albumin released from the cell surface can be detected in the washing solution by immunoelectrophoresis. We labelled albumin isolated by gel-filtration with ^{131}I isotope

according to Bocci's method (Bocci, 1963). Fig. 2 shows that the radioactivity of erythrocytes incubated with ^{131}I-albumin is well detectable after six washings (independently of the ATP content of the cells). Our albumin preparation was not free of fatty acids. It is known that unsaturated fatty acids get labelled with

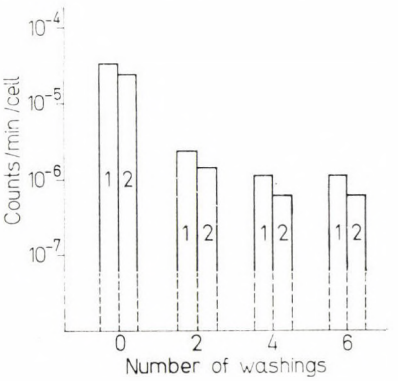

Fig. 2. Changes in the radioactivity of erythrocytes incubated with ^{131}I-albumin in the course of washings. 1: erythrocytes preserved for 6 weeks; 2: fresh erythrocytes

^{131}I and that erythrocyte membrane takes them over from albumin and incorporates them (Donabedian, Karmen, 1967). Therefore, we checked using the method of Jones and Gardner (1962) to what extent the ^{131}I-lipids were responsible for the radioactivity. We found that only about 15 per cent of the activity of erythrocytes washed six times could be extracted with lipid solvents. Thus the predominant majority of radioactivity derived from albumin. According to calculations based on the specific radioactivity of our albumin preparation, the quantity of albumin bound by the cells in the course of the incubation agreed in magnitude with the results of Ponder's measurements (Ponder, 1948) based on the decrease of the N-content of crystalbumin added to erythrocytes. The lower albumin concentration applied in our ATP regenerating experiment (see Fig. 1, tube 4) supplied a quantity lower than the albumin binding capacity of the cells, but the higher concentration (tube 3) was above this level.

Our experiments have led to the conclusion that — at least in the case of blood preservation — membrane fragmentation is not the cause but only a concomitant phenomenon of microsphaerocytosis. On the basis of recent ideas in biophysics a real symmetrical membrane fragmentation does not involve sphering, as a biconcave disk of shorter diameter might compensate for a decrease in surface. Fragmentation would be predisposing to sphaerocytosis only if it extended first of all on the equatorial membrane parts, richer in cholesterol and possessing a higher surface tension. According to the present state of biophysical research, the final osmotic pressure across the cell membrane is balanced

by the resultant of the elastic stress and surface tension and the sphering and antisphering agents act probably through altering surface tension (Fung, Tong, 1968). ATP may be necessary for the maintenance of the elastic (contractile?) structure of membrane, the mechanism of this process, however, is not yet elucidated. Albumin, named once "antisphering factor" by Ponder, might act through the alteration of surface tension. The residual albumin amount remaining on the erythrocyte membrane after washings represents only a few per cent of the starting value. It cannot be established whether this is indispensable for the partial shape regeneration occurring in crystalloid medium, when ATP level is restored. Complete regeneration, however, is a function of the albumin level.

To sum up, in the course of the regeneration of the ATP level of preserved erythrocytes, for the complete recovery of biconcave shape, the presence of a certain quantity of albumin in the medium or on the cell surface is needed. On the contrary, a lack of membrane fragments and the absence of their lipoid components do not inhibit shape regeneration.

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Mechanical Properties of the Flight Muscle of the Bee, II

Active Isometric Tension at Different Muscle Lengths

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Continuing their previous work on bee muscle the authors studied the changes of the active isometric tension at different lengths, in the course of gradual stretch and gradual release. The maximum tension corresponds to the resting length and it successively decreases reaching zero at a length which was earlier designated by us as the length corresponding to the end of the overlap of both kinds of filaments. An attempt was made to compare the results with those obtained on vertebrate muscle.

Introduction

In the first paper of this series (Garamvölgyi, Belágyi, 1968) we presented our observations on the resting elasticity of the wing muscle of the bee. This kind of muscle exhibits some properties which make it an object very suitable for mechanical studies. There is virtually no connective tissue and, as a consequence, the entirety of the mechanical properties is to be attributed to the myofibrillar substance (Pringle, 1967). The range of extensibility of this muscle extends to more than 300 per cent of the resting length. The length–tension curve of the resting bee muscle can be brought in connection with the changes of the cross-striation (Garamvölgyi, 1966) and it may supply valuable informations on the arrangement of the myofilaments.

The present paper deals with the active isometric tension of bee muscle in relation to the degree of the length changes and to the actual structure of the sarcomere.

Materials and Methods

For the present studies we used essentially the same experimental equipmen which was described in the first paper of this series (Garamvölgyi, Belágyi, 1968) The way of the preparation of thin muscle bundles was also the same. The muscles were fixed to steel hooks carried by the two opposite statives of the "Gleitmikro-manipulator" of Zeiss-Jena. The one hook was rigidly attached to the outside plate of the capacity transducer. The muscle was stretched by moving the opposite sled of the micromanipulator controlled by a micrometer screw permitting displace-ments of 0.01 mm.

After mounting the specimen we stretched the muscle with about 1 mm and attached 0.1 mm flexible platinum wire loops to its both ends. These wires served as stimulating electrodes. The muscles were immersed into a plexiglass vessel containing Pringle's solution. The block diagram of the device used throughout the experiments is shown in Fig. 1. After the mounting of the stimulating

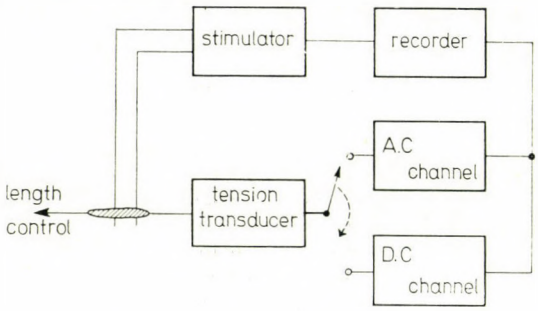

Fig. 1. Block diagram of the experimental equipment

electrodes the muscle was released and its resting length was roughly adjusted by means of the D.C. channel of the equipment (Fig. 1). As resting length we adopted the length at which resting elasticity was just perceptible (Garamvölgyi, Belágyi, 1968). In order to eliminate the tension changes following each individual stretch the active tension developed as a result of the stimulation was recorded through the A.C. channel. The appropriate time constant of the latter was 2 sec.

For stimulation we used a square-wave impulse generator. In most cases we applied short tetani of 0.2 sec duration. The amplitude of the stimuli was 20–50 V and their frequency varied from 50 to 200 Hz. The recording was performed with a BD-2 "Micrograph" ink recorder of Kipp–Delft with a chart speed of 600 mm/hour.

Results

The highest active isometric tension is found at (or just near) the adjusted initial length. This is probably due to the extremely short working distance of this muscle, the structural expression of which can be found in the short and not well-expressed I-bands. Thus the length tension diagram is highly asymmetric, in contrast to frog muscles in which the maximum force development corresponding to the resting length lies about half-way between the two zero values of the isometric force development.

In the course of the extension the force development steadily decreases reaching zero generally at a muscle length close to 2.5 mm (i.e. 100 per cent) elongation (Fig. 2, left). When using frequencies within the range of 50–200 Hz

we can roughly say that the higher the frequency the closer to 100 per cent elongation the tetanic tension reaches the value of zero in the course of its decrease.

When starting from the stretched state in the direction of the resting length the range of the active force development does not considerably differ from that measured in the case of successive stretch (Fig. 2 right). The tension increases

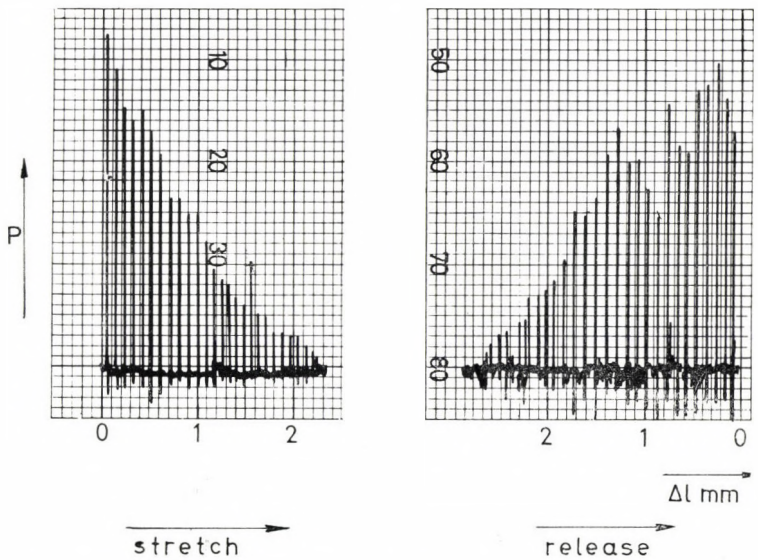

Fig. 2. Records of an experiment carried out in both directions (stretch and release) in steps of 0.1 mm

to about the resting length. There is often a slight shift of the maximum force development when measured in the opposite direction. This can be attributed either to an experimental error, or to a residual deformation. We do not want to deal in this paper with a possible contribution to this shift of polymer crystallization (Ernst, 1963).

The results of several experiments performed in both directions are summarized in Fig. 3. It can be clearly seen that there is a hysteresis-like difference in the course of the two curves. This effect can probably bear a relation to the differences in resting elasticity during approaching the same length from the two opposite directions. The stretch of the muscle causes a transient rise in the tension (e.g. Garamvölgyi, Belágyi, 1968), the tension reaching its equilibrium value in several minutes ("stress-relaxation"). If we measure the active isometric tension in the direction of increasing length, the muscles are usually stimulated when they still exhibit a resting tension higher than the resting equilibrium tension. In the direc-

tion of release the conditions are obviously different. The study of the relation of the active isometric tension to the actual state of the resting elasticity will be the topic of a further paper.

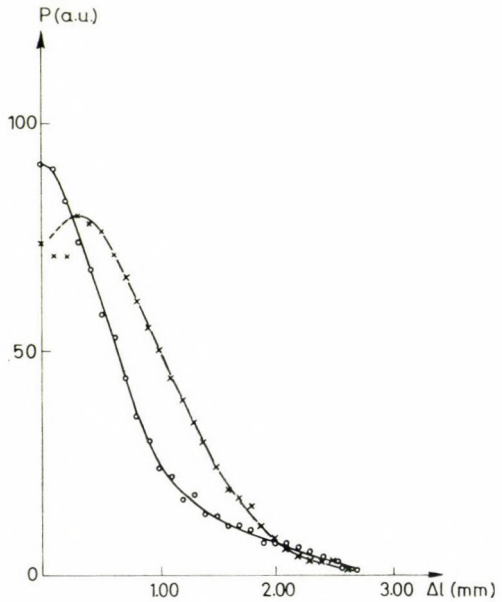

Fig. 3. Diagram constructed from the mean values of several experiments. Open circles: experiments performed by stretch (54 experiments). x-labels: experiments performed in the direction of release (31 experiments)

Discussion

In our previous work (Garamvölgyi, Belágyi, 1968) we found an abrupt change of the resting elasticity at a length corresponding to 100 per cent elongation. Reckoning with the length of the different bands of the striation (Garamvölgyi, 1966) we brought this length in connection with a sarcomere length at which both the "I-band length – sarcomere length" and the "A-band length – sarcomere length" curves exhibit sharp angles. This sarcomere length is close to the theoretical value of the end of overlap of the two kinds of filaments (no overlap length = A-band length + I-segment length), i.e. to about 5.5 μ. This length roughly corresponds to 200 per cent of the resting length. In the course of the present work we observed the zero value of the active isometric tension to be of about the same length (Fig. 3). The curve of the active isometric tension decreases to 2 per cent of its maximum value when reaching the length corresponding to 200 per cent of resting length (i.e. 2.5 mm elongation). Just at the same length the "length – resting tension" diagram exhibits a sharp angle (Fig. 4). This angle

has been suggested to mark the length of the minimum, if any, overlap (Garamvölgyi, Belágyi, 1968).

In frog muscle the no overlap length is 3.65 μ (Page and Huxley, 1963). According to the work of the A. F. Huxley's team (Huxley and Peachey, 1961, Gordon et al., 1964, 1966, 1966a, see also the paper of Podolsky, 1964) the tetanic

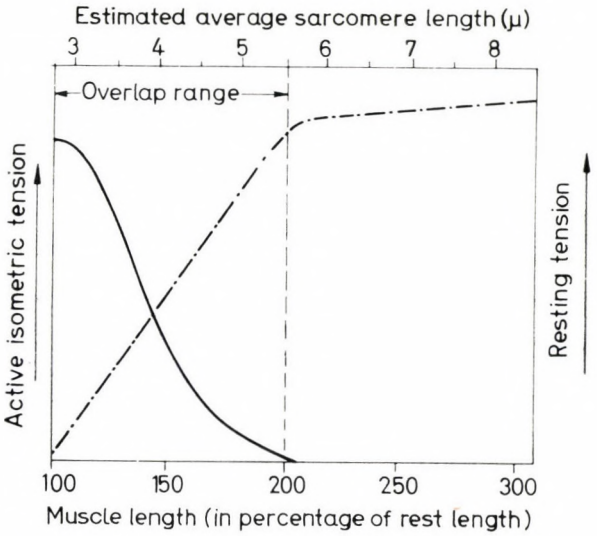

Fig. 4. Schematic diagram representing the relation between the courses of the different length–tension diagrams. Continuous line: "length–active isometric tension" diagram. Dotted line: "length–resting tension" diagram. (Partly after Garamvölgyi, Belágyi 1968)

tension virtually reaches zero at the length corresponding to this sarcomere length. Beyond this length there is a force development of only 2 per cent of the maximum tetanic tension, a value small enough to be attributable to residual irregularities. We also observed the same value of tension at 2.5 mm elongation. Thus our present work is in agreement with the results of the team of A. F. Huxley. This means that the length corresponding to the estimated end of the overlap suggested in our recent work (Garamvölgyi, Belágyi, 1968) can be established on experimental basis. The result of the determination of the no overlap point is compatible with the classical sliding model as well as with our sarcomere model (Garamvölgyi, 1965).

The fact that there is a steeply increasing resting tension up to the no overlap point and furthermore, that there is still resting tension beyond this point within a wide range, cannot be explained in terms of the classical sliding model (Huxley, 1953; Hanson and Huxley, 1955). On the other hand, the present results confirm that active isometric tension development seems restricted to the range of the

sliding movement. On this basis it seems possible to consider to what extent do the different constituents of the sarcomere contribute to the different aspects of the muscle function. At any rate, this coincidence of results seems to justify the efforts made in order to establish relationship between the physiological properties and the actual state of sarcomere structure.

The present findings confirm also all the previous statements, achieved on vertebrate muscle, on the development of maximum active tension at (or close to) the resting length (see Ernst, 1963). The specialized structural conditions of the insect flight muscle (e.g. short I-bands) do not influence the validity of the statement that the excited muscle develops maximum tension at its resting length.

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Mechanical Properties of the Flight Muscle of the Bee, III

The Reversibility of Extreme Degrees of Stretch

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Highly stretched muscles were gradually released and their length-tension diagram was taken up. The muscles were able to return to their resting length. The length-tension diagrams of the resting muscles exhibit a great hysteresis. Muscles were still capable of developing active isometric force after having been released several times following elongations of extreme extent. Even extensions up to 300 per cent of resting length did not cause irreversible tearings of the essential structural elements.

Introduction

The flight muscles of the majority of insects are highly inextensible (Pringle, 1967). The wing muscle of the blowfly (Calliphora sp.) exhibits irreversibly stretched zones at a very low degree of elongation (Hanson, 1956) and similar irreversible changes induced by passive stretch have been established in the flight muscle of waterbugs (Lethoceros sp.) (White, 1966).

Quite different conditions dominate in the flight muscle of the bee. Our earlier studies on the changes of the striation pattern (Garamvölgyi, 1965, 1966, 1966a) indicated a possibility of elongations up to more than 300 per cent of resting length and these observations have been confirmed recently by the first paper of this series (Garamvölgyi, Belágyi, 1968) dealing with the resting elasticity.

In the present paper we describe some experiments performed in order to elucidate the conditions of the structural and functional reversibility of extreme extensions. From the behaviour of the muscle in this respect it seems possible to conclude on its general architecture.

Materials and Methods

In these experiments we used the same experimental equipment which was described in the first two papers of this series (Garamvölgyi, Belágyi, 1968; Belágyi, Garamvölgyi, 1968). It was a gliding micromanipulator ("Gleitmikromanipulator" of Carl-Zeiss Jena) equipped with a capacity transducer coupled to a BD-2 "Micrograph" ink recorder of Kipp, Delft. The way of the preparation of muscle bundles was also presented earlier.

Before each experiment the starting length was adjusted and as resting length we adopted arbitrarily the length at which the resting elasticity became just perceptible. The stretch and the release of the muscles was controlled by a micrometer screw. The tension of the resting muscle was recorded through the D.C. channel and the active isometric tension through the A.C. channel of the equipment.

The extension happened in these experiments either gradually in steps of 0.5 mm, or continuously, with the speed of usual screwing movement. We obtained the same results in both cases. The study of the effect of different speeds was not the purpose of this work.

Results

Muscles stretched to 300 per cent of their resting length (i.e. $\Delta l = 5.0$ mm) were successively released. It is very interesting that as a result of the first release of 0.5 mm there was always an enormous fall of tension (Fig. 1). From this length

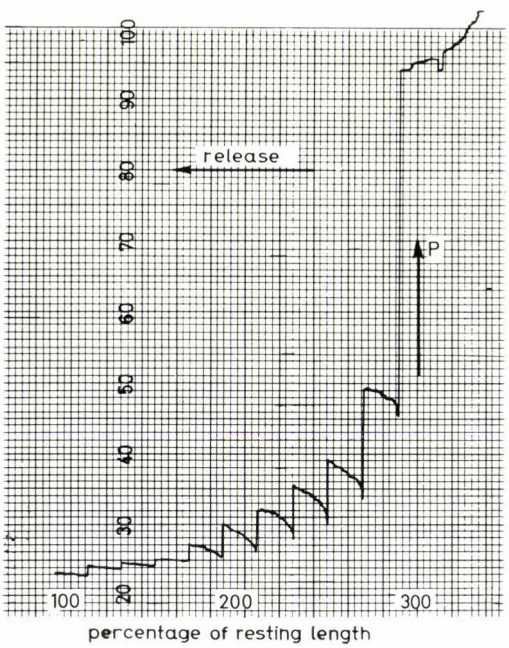

Fig. 1. Record of the gradual release of a highly stretched muscle (in steps of 0.5 mm

change on, the decrease of tension became smaller and smaller. From about 200 per cent of the resting length there was a very weak tension only. This means that the length-tension diagram of the resting, released muscle has a quite different course than the diagram taken up in the direction of stretch (Garamvölgyi,

Belágyi, 1968) (Fig. 2). It is very probable that in the course of release first the I-bands shorten, followed by the subsequent passive shortening of the stretched A-bands. At any rate, the muscles returned to their resting length, while still developing a very weak force within the second range of their release.

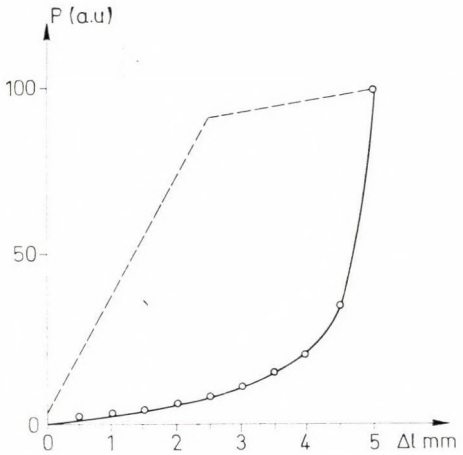

Fig. 2. Length-tension diagram constructed from the mean values of 14 experiments performed in the direction of release (lower curve) compared with the length-tension diagram taken up in the direction of stretch (Garamvölgyi, Belágyi, 1968) (upper curve)

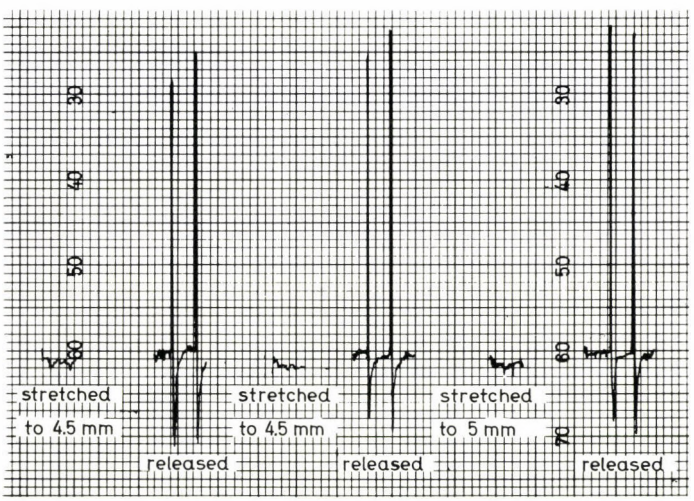

Fig. 3. Active isometric force development of a muscle in the course of repeated stretch and release, stimulated twice after each length change (starting from the left). Note the lack of force development in the stretched state

It is to be noted that within the first phase of the curve (down to about 200 per cent of resting length) the tension reaches its equilibrium value after several minutes of a slow increase of tension (Fig. 1), which represents probably the reverse phenomenon of the stress relaxation.

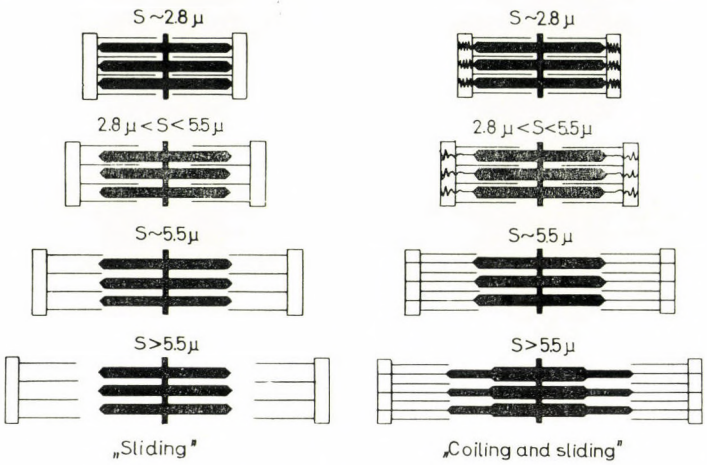

Fig. 4. Diagrammatic representation comparing the structural basis of the sliding and of the "coiling and sliding" model constructed for different sarcomere length ranges. Top: virtually total overlap, 2nd from the top: range of the sliding movement, 3rd from the top: no overlap point, bottom: range beyond the no overlap point

When stretched to 250–300 per cent of the resting length (i.e. far beyond the "no overlap point" corresponding to about 200 per cent of resting length) the muscles did not develop any measurable active isometric tension (Belágyi, Garamvölgyi, 1968). When released to their resting length and stimulated they were able to force development once more. This procedure could be repeated several times, and even after elongations of 200 per cent (i.e. up to 300 per cent of resting length) the released muscles were able to carry on their function obviously in a normal way (Fig. 3). This means that extreme degrees of stretch did not cause serious tearings of the myofibrillar structure.

Discussion

Our present results demonstrate that there are considerable differences in the mechanical properties of the bee muscle and of the muscles of other insects studied. In fly and waterbug muscles the maximum reversible elongation causes only an insignificant decrease of the degree of the overlap, while in the case of the bee the overlap might be reduced to virtually zero. Moreover, muscles can be elongated beyond this length within a wide range dominated by the elongation

of the A-band. Notwithstanding, these muscles are able to return to their resting length when released. This fact seems to exclude the existence of any irreversibly stretched zone in this muscle. It is true, however, that there is a marked hysteresis of the length-tension diagram, an indication of some residual structural consequences of the elongation (Ernst, 1963).

Neither the resting elasticity, nor the reversibility of stretch can be interpreted by the sliding model without the assumption of suplementary structures, of a set of filaments running from Z-line to Z-line. Some authors (e.g. Ernst, 1963) assumed the filaments to be continuous through several sarcomeres, i.e. to cross the Z-lines without interruption.

Our sarcomere model (Garamvölgyi, 1965) is, in some respects, also based on a morphological continuity, for we assume the C-filaments (Auber, Couteaux, 1962, 1963; Garamvölgyi, 1963; Pringle, 1967) to represent mechanical connections between the Z-line and the myosin filaments. On the basis of electron microscopic (Garamvölgyi, 1963, 1965) and polarizing microscopic (Garamvölgyi, 1966a) observations we supposed the material of the C-filaments to be coiled inside the Z-lines in the case of unstretched resting muscles (Garamvölgyi, 1968. Garamvölgyi, Belágyi, 1968). As a consequence, we assume a structural mechanism more complex than simple continuity. Our sarcomere model could be designated as a "coiling and sliding" model, which might be compared with the sliding model in Fig. 4.

Thus the phenomena described in this paper could be explained on the basis of our model.

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Use of X-ray Diffraction Method in Investigations on Mineral Substances of Bone and Callus

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The authors have employed Debye—Scherrer's method in investigations on the mineral substances of the bone. Besides the characteristic apatite X-ray diffraction lines, also lines of $CaHPO_4 \cdot 2H_2O$ and $CaHPO_4$ were found in some cases. The crystals constructing the mineral substance of the bone show different forms.

The analysis of the developing callus by X-ray diffraction methods and its comparison with the artificial formation of apatite have rendered possible to put forward a hypothesis of the natural formation of apatite. The developing callus has not yet been analyzed by other investigators with this method. According to this hypothesis the apatite of callus and bone developes in identical or similar steps as does artificial apatite.

According to the present experiences X-ray diffraction, when used under appropriate conditions, seems to be a very suitable method to follow some biodynamical processes of the bone and callus.

Introduction

De Jong (1926) was the first to perform X-ray diffraction investigations of mineral substances of the bone. He identified the crystalline structure of the mineral substance and recognized that its diffraction pattern was almost the same as that of the fluoroapatite. In the course of further investigations it was established that the structure of the mineral part of the bone mostly resembled the hydroxylapatite. The aim of these investigations was the determination of the size and direction of the crystals of the mineral substance. Several kinds of methods have been employed by various authors in the course of these investigations.

Since the size of the granules is extraordinarily small in the case of bone it was necessary to develop a microdiffraction technique for the investigations, besides using the broad-angle diffraction method. Such technique was employed, among others, by Engström and Finean (1953), Spark, and others.

The small-angle diffraction in a well-oriented system gives informations about the dimension and orientation of the particles. Such a method was used, for example by Finean (1953) who described a simple small-angle camera as well.

Mosebach (1965) employed the diffractometric method and using Debye's formula he determined the size of the elementary crystal granules from the widening of the lines.

Recent investigations attempt to elucidate the dynamics of the processes taking place in the bones in contrast to earlier investigations which were static in character. Some of these investigations analyse the changes in crystalline structure of the mineral substances after introduction into, or withdraw from the organism of certain substances. No account is given by the authors about larger series either in relation to this subject or to the former one.

First of all, the fluorine is worthy of mention out of the substances administered. The beneficial effect of fluorine administration in preservation of the intactness of the teeth has been described by several authors; as it is known the health of teeth essentially depends on the intact character of the mineral substances. According to Posner and Eanes (1963) the effect of fluorine on the crystalline structure manifests itself in the enlargement of the size of the crystals on the one hand, and in the development of a more perfect crystal structure of apatite on the other. Posner et al. (1965) have observed also a faster development of the crystals under the effect of fluorine. Gron et al. (1966) have also observed a higher degree of crystallization after fluorine treatment of osteoporosis.

Muller et al. (1966) have observed alterations of opposite direction in vitamin D deficiency. The development of crystals was slower and the degree of crystallization lower than under normal conditions.

Also the experiments of Posner et al. (1965) in which the degree of crystallization was brought into connection with the age can be regarded as investigations of dynamic character.

The aim of the investigation

Our own investigations were performed according to the Debye – Scherrer method. The aim of the experiments was as follows:

1) Analysis of the X-ray diffraction pattern of deep-frozen and desantigenized human and bovine bones, with special regard to the shapes of crystals and to the non-apatite lines of the pattern.

2) Description and interpretation of certain crystallographic characteristics of post-fracture callus formation and its comparison with the artificial formation of apatite.

In the course of the investigations a total of 41 X-ray diffraction patterns have been analyzed.

I. Roentgen diffraction analysis of human and bovine bones

Materials

The conserved bone was supplied in each case by the Department of Tissue Conservation of the National Institute of Traumatology. The measurements were carried out on preparations obtained partly from deep-frozen human tibia and partly from desantigenized tibia corticalis of the calf. Deep-frozen bones were analyzed in eight cases and desantigenized bones in eight further cases.

Prior to the roentgen diffraction analysis the bones were subjected to lyophilization for six days. Thereafter the substance was pulverized as fine as possible in an agate mortar. The procedure led to a material as dry as no delubrication was necessary in any case.

Technique

Roentgen diffraction investigations were performed by means of a Müller-Mikro 60 type radiographic apparatus, operated by an iron anticathode Phönix tube. The beta ray was not filtered out of the iron radiation because any decrease of energy caused by the filter had to be avoided. The samples to be analyzed were placed in a capillary tube of 0.3 mm diameter and of 0.02 mm wall thickness made of Movilite. During exposure the preparations were rotated at a speed of 10 r.p.m. in order to increase reflection probabilities. The time of exposure was determined by the amount of crystalline part contained in the substance to be analyzed. This time generally extended from 5 to 10 hours.

The reflections were fixed on a Gaevert Struktur X film and, after developing, we measured them out by a comparator of 0.05 mm accuracy. The lines of the measured records were compared with the values found in the literature (ASTM card-index system) or with our own internal standards. It is the advantage of the Debye–Scherrer powder recording method employed by us that only reflections from the crystal lattice are observable.

Results

The roentgen diffraction patterns of the deep-frozen human bone and the desantigenized calf bone do not reveal any essential difference. Several Debye–Scherrer's lines were observable on the records. The number of lines is proportional to the amount of crystal substance contained in the samples, for the records were taken under identical conditions. As a consequence, we could generally measure out 10 to 12 lines on the records, in contrast to the 35 lines of the record made of natural apatite.

Some of the lines detectable on our records originate in the lattice plane of hkio or 0001 index, while others in the lattice plane of hkil type of index. The lines appearing on the records are gathered so that, on certain types of records, the reflections of hkio and 0001 types are either completely missing or, if present, they only appear in a rather subordinate way, while in the reflections appearing in another group of samples it is the reflections of hkil type which are overshadowed (Table I, Column 1, Table I, Column 2).

On this ground it may be assumed that the development of the apatite crystals in the bone does not take place with identical arrangement of the lattice plane; the predominating form is the combination of prism and basal pinacoid in certain cases, while the bipyramid in others.

Table I

Column 1 Values of roentgen diffraction showing the dominance of the combination of prism and basal pinacoid in apatite crystals			Column 2 Values of roentgen diffraction contain only the pure lines of bipyramid index of apatite crystals		
d_{hkl} kX	Intensity	hkil	d_{hkl} kX	Intensity	hkil
4.56	w		4.14	i	
4.10	vi	2020	3.69	m	
3.70	m		3.36	w	
3.40	m	0002	2.77	i	1122
3.07	m	2130	2.28	m	2132
2.79	i	1122			
2.52	w	3030			
2.25	w	3140			
2.12	i	3141			
1.93	w	2242			
1.83	w	2133			
1.719	i	0004			

Abbreviations in Tables I to IV: i = intense, vi = very intense, m = medium, w = weak, vw = very weak

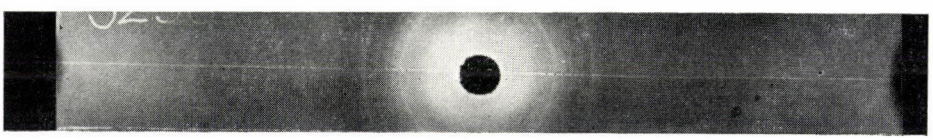

Fig. 1

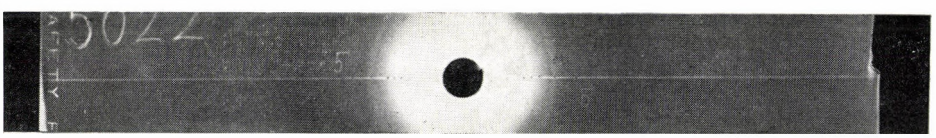

Fig. 2

In seven roentgen diffraction records a few lines were detected besides the lines of apatite which could not be identified with the lines of apatite. These lines did not show identity with the lines of the record taken of lyophilized grinded collagen because this latter only consisted of an amorphous ring. The sharp lines detected by us indicate a well-defined crystalline structure. In the course of their identification it became known that they corresponded to strongest and thus most characteristic lines of $CaHPO_4$ and $CaHPO_4 \cdot 2H_2O$. According to the present state of our experiment the lines of the two substances do not appear simultaneously on the records. Table II and Fig. 3 – 5 show the characteristics of roentgen diffraction of mineral substances consisting purely of apatite, of the combination of apatite and $CaHPO_4 \cdot 2H_2O$ and of the combination of apatite and $CaHPO_4$.

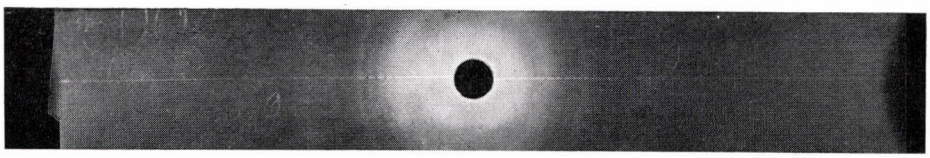

Fig. 3

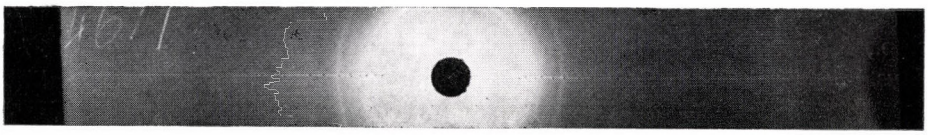

Fig. 4

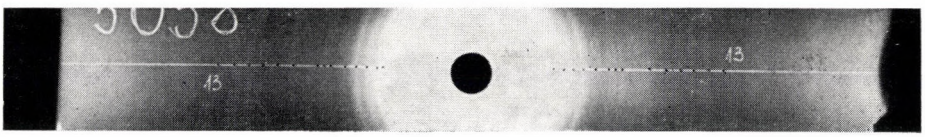

Fig. 5

Table II

Column 1 Identifiable lines of roentgen diffraction pattern only show the presence of apatite (a)		Column 2 Lines of roentgen diffraction show the presence of $CaHPO_4 . 2 H_2O$ (d) besides apatite (a)		Column 3 Lines of $CaHPO_4$ (h) also appear besides apatite (a) in roentgen diffraction pattern	
d_{hkl} kX		d_{hkl} kX		d_{hkl} kX	
4.13	a	4.57	d	3.66	h
3.70	?	4.18	d	3.36	h
3.40	a	3.69	d	3.06	a
3.07	a	3.46	a	2.77	a
2.79	a	3.10	a	2.52	a
2.29	a	2.79	a	2.29	a, h
1.94	a	2.43	d	2.19	h
1.83	a	2.25	a	2.10	a
1.72	a	2.00	a	1.83	a
		1.835	a	1.70	?
		1.725	a	1.59	h
				1.50	h
				1.44	a
				1.35	h

II. Analysis of the callus by roentgen diffraction

The development of apatite in callus was traced by roentgen diffraction analysis. Such investigations have not been performed up till now to our knowledge.

For the purpose of obtaining callus fracture was performed on 10 inbred 3 months old guinea-pigs, and on 6 similarly 3 months old chinchilla rabbits. The fractures were made on the legs of the animals.

Two guinea-pigs were killed on the 7th, 10th, 14th, 21st and 28th days, resp., following the fracture, and 3–3 rabbits were killed on the 14th and 28th days. The developed callus was carefully removed from the original bones, taking care that no original substance of bone should get into the preparation. Each callus was subjected to lyophilization for six days, then pounded in agate mortar and analyzed by the Debye–Scherrer procedure already described.

Results

It should be mentioned in connection with the evaluation of the results that, on the basis of the large number of phase analyses performed by one of us by means of the Debye–Scherrer method, we came to the conclusion that the amount of constituents present in the phase mixtures is proportional not only to the intensity but also to the number of the appearing lines.

The lines mentioned in the literature as the most intense ones did not appear always as the most intense lines. On the other hand, the less intense lines appeared with greater intensity in certain samples in the course of the present investigations. Owing to this, it was the number of the appearing lines which was taken into consideration for registration of the crystallization process. The reason for the changes of intensities is not known at present (deformation of lattice plane, polymorphous alteration?) and it needs further investigations.

One of the analytical series of the callus of the guinea-pig is shown below in details:

In the 7 days old callus no lines of crystalline substance are yet distinguishable. The crystals are here either not yet developed or their number is as low that the demonstration is not possible with our method (Fig. 6).

Three lines could be measured out in the callus which was removed on the 10th day. This means the initial stage of crystal formation. Two of these lines are apatite lines, while the third is the line of $CaHPO_4 \cdot 2H_2O$. Their ratio is 2 : 1 (Table III/2, Fig. 7).

In the 14 days old callus four identifiable lines could be determined. Out of these, two are apatite lines and two are $CaHPO_4 \cdot 2H_2O$ lines. The ratio in this case is 2 : 2 (Table III/3, Fig. 8).

On the 21st day nine lines became visible. Their distribution was as follows: the number of apatite lines was 6, and the number of $CaHPO_4 \cdot 2H_2O$ was 3. Thus, the ratio was 6 : 3 (Table III/4, Fig. 9).

Table III

1	2		3			4			5		
d_{hkl} kX	Int.		d_{hkl} kX	Int.		d_{hkl} kX	Int.		d_{hkl} kX	Int.	
3.38	i	d	5.79	i	?	4.23	w	d	3.78	w	a
3.06	m	a	4.23	vi	d	3.78	m	a, d	3.42	m	a
2.78	vi	a	3.38	vw	d	3.44	i	a	3.08	m	a
			3.08	vw	a	3.08	m	a	2.78	i	a
			2.78	vw	a	2.79	vi	a	2.26	vw	a
						2.296	w	a	1.944	vw	a
						1.956	vw	?	1.833	vw	a
						1.841	vw	a			
						1.718	vw	a			
						1.455	vw	d			

1. No line can be measured in 7 days old callus of guinea-pig
2. Roentgen diffraction data of 10 days old callus of guinea-pig
3. Roentgen diffraction data of 14 days old callus of guinea-pig
4. Roentgen diffraction data of 21 days old callus of guinea-pig
5. Roentgen diffraction data of 28 days old callus of guinea-pig

Abbrevations used: a = apatite, d = $CaHPO_4 \cdot 2\,H_2O$, Int. = Intensity, i = intense, vi = very intense, m = medium, w = weak, vw = very weak

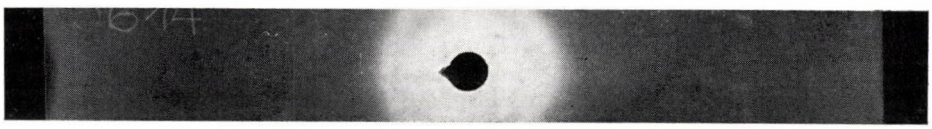

Fig. 6

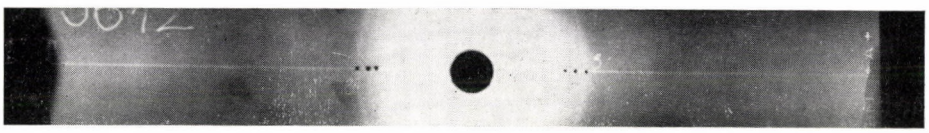

Fig. 7

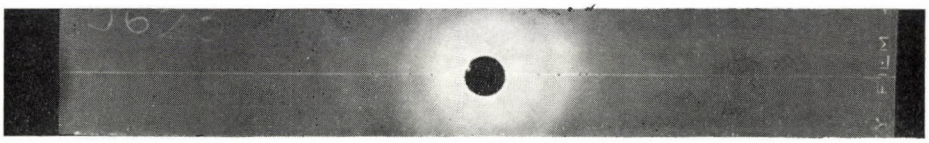

Fig. 8

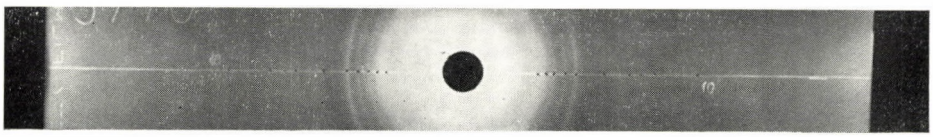

Fig. 9

The 28 days old callus had seven apatite lines but it did not contain any $CaHPO_4 \cdot 2H_2O$ line. The ratio in this case was 7 : 0 (Table III/5, Fig. 10).

The callus developing on the legs of the rabbits after the fracture was removed at the end of the 2nd and 4th week. In the first group either an amorphous ring was found without signs of crystallization or a combination of apatite and

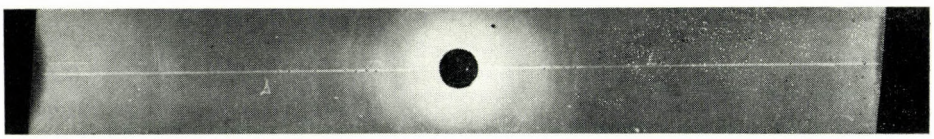

Fig. 10

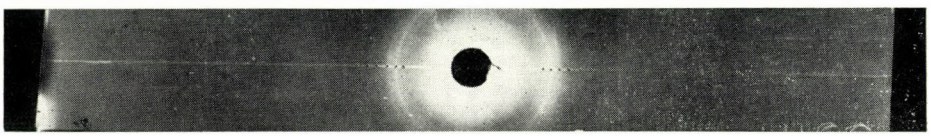

Fig. 11

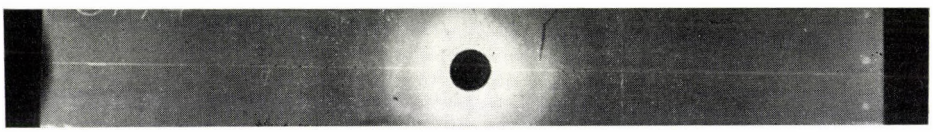

Fig. 12

$CaHPO_4 \cdot 2H_2O$ in a ratio of 2 : 5 (Table IV/1, Fig. 11). In the callus removed at the end of the fourth week only the lines of apatite were detectable (Table IV/2, Fig. 12).

Table IV

1 Roentgen diffraction pattern of developed callus 14 days after fracture in rabbit			2 Roentgen diffraction pattern of developed callus 21 days after fracture in rabbit		
d_{khl} kX	Intensity	Substance	d_{hkl} kX	Intensity	Substance
4.57	m	d	3.44	i	a
4.23	vi	d	3.08	m	a
3.73	i	d	2.78	vi	a
3.36	w	d	2.26	w	a
3.08	vw	d, a	1.926	vw	a
2.77	m	a	1.828	vw	a
			1.710	w	a

a = apatite, d = $CaHPO_4 \cdot 2H_2O$

III. Development of artificial apatite

The roentgen diffraction characteristics of the development of artificial apatite was also studied in three series parallel with the development of the apatite of callus. Calcium-phosphate compounds with apatite structure are produced on large scale by the interaction of aqueous solutions containing Ca^{++} ions and PO_4^{3-} ions. The investigations of Bidló and Fodor (to be published) demonstrate that, depending on the circumstances, three kinds of substances of crystalline structure are obtained in the course of artificial apatite production. These substances are:

$CaHPO_4 \cdot 2H_2O$	(brushite)
$CaHPO_4$	(monetite)
$Ca_5(PO_4)_3 \cdot F \cdot OH$	(apatite)

The crystalline structure of brushite is monoclinal, the monetite's structure is triclinal and apatite has a hexagonal structure.

According to the above-mentioned analysis artificial brushite can be obtained by mixing diluted $CaCl_2$ (0.5 mole) and diluted $(NH_4)_2HPO_4$ solutions (0.5 mole)

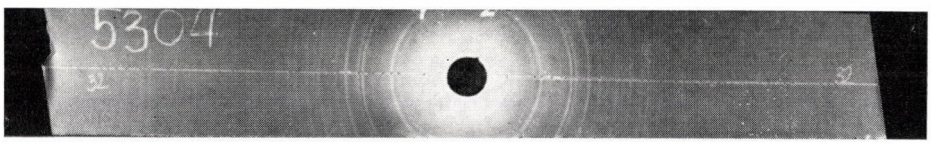

Fig. 13

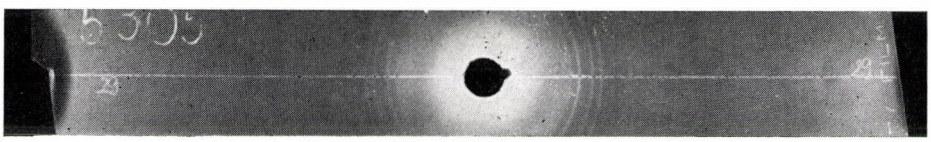

Fig. 14

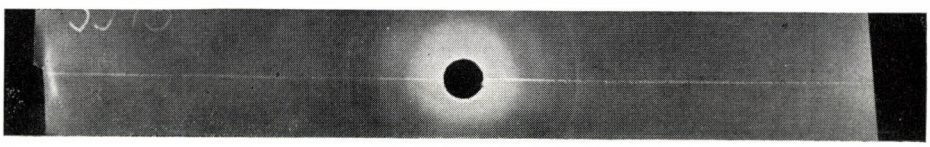

Fig. 15

and stirring them slowly but vigorously at room temperature. The white precipitate formed is dried in vacuum exsiccator at 60°C. The substance is crystalline and shows the structure of $CaHPO_4 \cdot 2H_2O$ either when analyzed by roentgen diffraction or when studied by derivatographic methods (Table V/1, Fig. 13). The substance decomposes endothermically and undergoes a transformation into $CaHPO_4$ at a temperature of 180°C (Table V/2, Fig. 14).

Artificial monetite can be produced in a direct way too, if relatively concentrated (3 mole) solutions are mixed together at a very quick rate after preheating up to 90°C. The white precipitate formed is filtered and dried at 150°C. The substance obtained shows the structure of $CaHPO_4$ both in roentgen diffraction records and in derivatograms. When heated to 460°C the sample loses 6 per cent of its weight and its structure is transformed into apatite (Table V/3, Fig. 15).

Table V

Phases of artificial apatite formation

1 $CaHPO_4 \cdot 2\,H_2O$		2 $CaHPO_4$		3 Roentgen diffraction data of apatite	
d_{hkl} Å	Intensity	d_{hkl} Å	Intensity	d_{hkl} Å	Intensity
7.60	vi				
4.64	m				
4.23	vi				
		4.16	i		
				4.05	m
3.75	w			3.78	m
		3.66	m		
				3.44	i
3.38	w	3.36	vi		
3.23	vw				
3.06	i	3.09	vw	3.08	m
2.93	m	2.93	vi		
				2.775	vi
		2.708	i	2.715	i
2.62	i			2.629	m
2.44	m	2.46	w		
				2.264	w
		2.23	w		
2.17	m	2.18	vw		
				2.149	w
2.10	w				
2.01	m	2.02	vw	2.01	vw
		1.980	vw	1.944	i
1.892	m	1.903	w	1.890	w
		1.843	m	1.840	i
1.828	m				
				1.806	w
		1.789	vw	1.781	w
1.723	w				
		1.719	m	1.719	w
				1.644	vw
1.616	w				
1.562	w				
1.528	w				
1.463	w				

(Further data are not published.)

Discussion

1. The analysis of roentgen diffraction patterns of the bone and callus on the one hand, and of the artificial apatite on the other called our attention both to similarities and differences.

In addition to apatite the same compounds could be detected both under natural and artificial conditions. These compounds were $CaHPO_4 \cdot 2H_2O$ and $CaHPO_4$ which, in the case of artificially produced apatite could be revealed at different stages of production. In some of the records made of the bone the one compound or the other appeared besides apatite, while at the early stages of callus development $CaHPO_4 \cdot 2H_2O$ appeared in high proportion.

On the basis of these observations the question arises whether $CaHPO_4 \cdot 2H_2O$ and $CaHPO_4$, compounds found in natural state besides apatite, do not contribute, in certain phases to the formation of natural apatite in a way similar to that seen in the course of artificial apatite production. It is known that the living bone is the place of continuous changes, syntheses and decompositions. Apatite formation occurs in some given and examined volume with a certain probability all the time. With these considerations in mind, it would be possible to give an explanation for the presence or absence of compounds observed besides apatite in the developed bone. If roentgen diffraction analysis is performed in such a part of the bone where significant apatite formation is in progress $CaHPO_4 \cdot 2H_2O$ or $CaHPO_4$ lines also appear besides apatite, but only one or the other of them, just like in the case of apatite formation in laboratory. If only apatite lines are observed it can be assumed that there was no apatite formation in the volume analyzed at the given time, or the formation rate was as low as the roentgen diffraction method was not capable of demonstrating it. This method gives information about substance quantities only if the latters amount at least 10 per cent of the volume analyzed.

In the explanation of the roentgen diffraction pattern of callus our assumption is supported by the behaviour of the $CaHPO_4 \cdot 2H_2O$ phase the ratio of which is greater in younger callus and later decreases; for increased apatite formation the increase of the precursors is namely also necessary at the early stages.

On the other hand the phase of $CaHPO_4$ was not detected in the callus. This may refer to further unknown characteristics of callus formation but it can be simply a coincidence because only a few analyses have been performed on material concerning 4 weeks of callus formation and the immense number of phases of the transformation process.

One of the most significant difference between the development of natural and artificial callus lies in the energy supply needed for the process of transformation. During the production of artificial apatite this can be ensured by temperature not realizable under natural conditions. It is possible that nature ensures this energy by enzymes or enzyme systems which are unknown because our knowledge about the enzymology of bones is not yet sufficient enough.

2. Taking into consideration our experiences thus obtained and also the theoretical reasonings we think that the method of roentgen diffraction seems to be an adequate procedure for studying certain biodynamical processes of the bone. With the aid of this method very small quantities of newly-formed mineral substances can be detected and traced. The roentgen diffraction pattern shows only an instantaneous exposure of one single phase of the process, thus, the simultaneous evaluation of a great number of records is necessary to gain information about the whole process.

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Effect of Antibiotics on the Ion Exchange of Bacteria*

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It has been shown in the course of our investigations that a chloramphenicol treatment decreased the rate of ^{42}K exchange of bacteria, and it diminished the calculated permeability constant to 45 per cent of the value obtained in control cells. This ion transport decreasing effect of chloramphenicol is assumed to be due to disconnection of the energy source of transport, i.e. of the chemical energy of intermediates arising during the breakdown of ATP.

The joint effect of streptomycin and chloramphenicol on ^{42}K-exchange of bacteria has been also investigated. It has been established that the simultaneous application of equal concentrations of the two antibiotics do not change the rate of ion exchange. According to our supposition a competitive antagonism exists between chloramphenicol and streptomycin.

Introduction

One of our previous papers (Tamás, Szőgyi, 1966) described investigations concerning the ^{42}K transport of sensitive and resistant E. coli cells treated with streptomycin. It was established that the efflux of ^{42}K was faster from sensitive than from resistant cells or from bacteria not treated with streptomycin. The permeability constants have been calculated and it was found that the permeability for ^{42}K ions of streptomycin-treated sensitive E. coli cells increased by 61.3 per cent. In our opinion streptomycin injures the walls of sensitive cells, and consequently it changes the barrier of permeability.

Several investigations have been reported in the literature (Gale, Folkes, 1953; Brock, 1961; Ochoa, 1963; Parthier, 1965) which showed chloramphenicol to be a specific inhibitor of protein synthesis. The fact that there is a correlation between protein synthesis and potassium turnover prompted us to investigate in the present experiments the effect of chloramphenicol on the efflux of ^{42}K from bacteria.

Several observations point out that the bactericid effect of streptomycin exerted on bacteria in vitro is prevented by chloramphenicol (Anand, Davis, 1960; Hurwitz, Rosano, 1962; Plotz, Davis, 1962; White, 1964; Vasquez, 1964, 1966). In further investigations we studied the question whether chloramphenicol is capable of changing the increasing effect of streptomycin on ^{42}K efflux as well.

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Methods

E. coli B bacterium was used for the investigations. The bacterium cells cultured in agar medium for one night at 37°C were inoculated from this medium into 500 ml of M^9 synthetic culture medium three hours before starting the experiment. The cells, which were in the logarithmic phase of growing, were centrifuged with 8000 cps and collected in 40 ml of M^9. Thereafter, 300 μCi of ^{42}K was given to the suspension. After one hour incubation the cells were centrifuged, the active culture fluid was poured down, the residual bacteria were washed twice with 20 – 20 ml of M^9 culture medium and were suspended in 40 ml of M^9. The suspension obtained was divided into two parts. One portion served as control; to the other part 60 μg/ml of chloramphenicol were added in the first series of the experiments and 30 – 30 μg/ml of streptomycin and chloramphenicol respectively, in the course of further experiments. The amount of antibiotics, added to the suspension did not perceptibly decrease the number of living cells. 3 – 3 ml samples were taken at appropriate intervals, poured on G-5 type bacterium filters and filtrated under reduced pressure. The bacteria left on the filter were washed twice with 10 – 10 ml of M^9 and the activity of the residue was measured. The activity of ^{42}K adsorbed at the pores of the filters was eliminated by washing the bacterium cells from the filter and by measuring the activity again. The difference between the two results was taken for the activity of the labelled ions remaining in the cells. The cells were counted under microscope before each experiment and the number of living cells was controlled by inoculation. Knowing the number of bacteria we determined the mass of them and calculated the specific activity.

Results

Fig. 1 shows the summarized results of 10 – 10 measurements concerning the efflux of ^{42}K from E. coli cells treated and non-treated with chloramphenicol. The specific activity, or more precisely, the value of cpm/g proportional to it, was plotted against time (measured in minutes) in a semi-logarithmic scale. Fig. 1 shows that, owing to the effect of chloramphenicol, the exchange of ^{42}K slows down and the calculated permeability constant decreases by 45 per cent when compared with control cells:

$$k_K = (4.69 \pm 0.28)\, 10^{-7}\, \frac{\text{cm}}{\text{sec}},$$

$$k_{CAP} = (2.59 \pm 0.17)\, 10^{-7}\, \frac{\text{cm}}{\text{sec}}.$$

When chloramphenicol and streptomycin were added at the same time and at identical concentration level (30 – 30 μg/ml) to the E. coli culture previously incubated with ^{42}K the rate of ion efflux remained unchanged. Thus, under

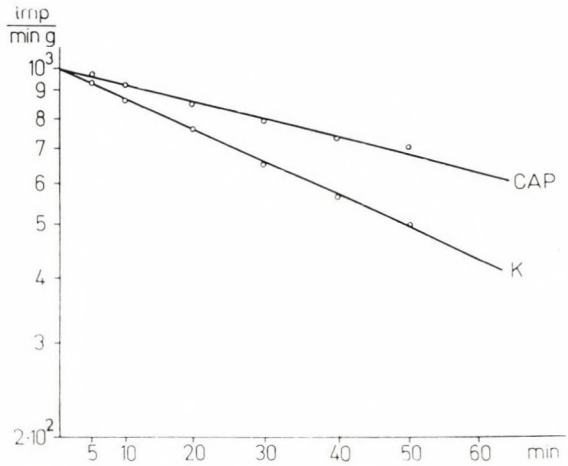

Fig. 1. Efflux of ^{42}K from E. coli cells treated (CAP) and non-treated (K) with chloramphenicol

such circumstances chloramphenicol, so to say, "prevented" the ^{42}K exchange-increasing effect of streptomycin. If chloramphenicol was added 10 minutes after starting the streptomycin treatment the permeability constant for ^{42}K increased by 32.7 per cent. When streptomycin was given to the pretreated chloramphenicol-culture the ion exchange decreased by 18.5 per cent.

In the further experiments we changed the proportional concentration of the two kinds of antibiotics. If the concentration of chloramphenicol was five times higher than the concentration of streptomycin the liberation of ions decreased by 32 per cent. On the other hand, if the concentration of streptomycin was raised on its fivefold the ion transport increased by 85 per cent.

Discussion

As it has been mentioned in the introduction, chloramphenicol is a specific inhibitor of protein synthesis. Nevertheless, both according to our conclusions and those of other authors (Tamás, Szőgyi, 1967; Hanson, Hodges, 1963; Sutcliffe, 1960) the energy source of active transport is the chemical energy of the intermediates arising during the decomposition of ATP. Presumably, chloramphenicol interferes with a reaction which influences the synthesis of high-energy phosphate compounds. Thus, in all probability, the ion transport-decreasing effect of chloramphenicol in bacteria can be accounted for to the disconnection of the energy source necessary for the transport.

To explain the experimental results concerning the simultaneous effects of the two antibiotics we assume a competitive antagonism to exist between

chloramphenicol and streptomycin. The two antibiotics given in equal concentration are most probably adsorbed on the surface of, or bound to, the cell in the same proportion, and thus their effects on ^{42}K exchange are mutually compensated. This assumption is also supported by experiments in which the addition of certain antibiotics to the bacterium culture was carried out at different times and different concentrations were used.

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Responses of a Possible Active Small-signal Model for Excitable Systems

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Recorded responses and calculated types of equilibrium of an active RC network are shown in order to demonstrate that networks of this sort can produce all the main types of linear responses of excitable biological systems. Moreover, the active networks are not merely a possibility. The neural system appears to be able to exhibit unstable oscillations in the linear region about its resting state, thus it can be regarded, even in this linear region, to be *active* in circuit-theoretical sense, or more precisely, to be *an equivalent* of an active network. This has importance in the biophysical processes involved.

Introduction

Electrically, the excitable biological systems are nonlinear, but they respond as linear systems to sufficiently small subthreshold stimuli, i.e. the relation between such stimuli and the response is described by linear differential equations. (The threshold is meant here with respect to the propagated spike potential.) On this basis we may talk about "linear subthreshold responses" or "linear operating region" of the neural system (by operating region we mean a certain range of operation or the set of states in which a certain mode of operation holds; Thaler, Pastel, 1962). The existence of the linear region is a general property of the systems. In technical terms: the system works within a sufficiently close neighbourhood of an operating point as a linear system. This property is familiar in the engineering, e.g. this is the case with any amplifier; from mathematical point of view it is obvious in all cases when the system's equations contain only analytical functions. (See also Andronov et al., 1966.) — Thus one can design linear lumped parameter equivalent networks for the excitable systems, which apply only to the linear region about their resting state (i.e. the linear subthreshold responses). Such "small-signal" equivalents are useful to answer many questions and have been commonly employed in the literature (Cole, 1941; Knutsson, 1964; Chandler et al., 1962; Lewis, 1965). Hodgkin and Katz ascribed the subthreshold electric changes at stimuli of less than 30 p.c. threshold to a passive RC circuit, which is a linear system (nerve fibres of Carcinus maenas, Hodgkin, 1938; frog gastrocnemius muscle, Katz, 1937; frog muscle fibre, Katz, 1948). The electric changes in this region were called local potential (Katz, 1937) or passive charging process or passive polarization (or depolarization) process (Hodgkin, 1938, 1948). —

Nevertheless, a passive RC circuit is not the adequate small-signal equivalent to model all the main types of local potentials observed experimentally, such as: 1. the non-oscillatory responses; 2. the stable oscillatory responses; 3. the unstable oscillatory responses. The following qualitative small-signal model has been constructed with the purpose of producing, by the same lumped network, all these types of response by varying one parameter.

Results

1. The system considered (an active RC two-port network) was closely linear up to 0.15 V output amplitude. Its idealized representation is shown in Fig. 1. By changing the gains or other parameters this system may be stable and

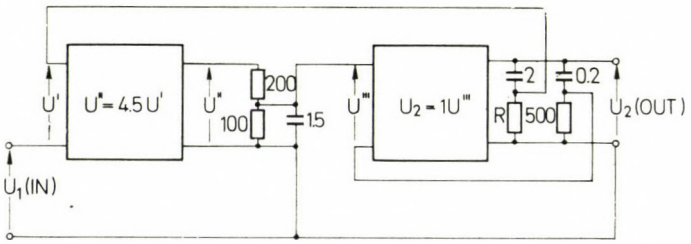

Fig. 1. A linear active network. The squares are block-representations of voltage-amplifiers, the numbers denote voltage-gain, ohms and microfarads, respectively

unstable non-oscillatory, and stable and unstable oscillatory (for "stability" and "instability" see: Thaler, 1955). We have recorded the responses of the real system (Fig. 2) at the parameter values given in Fig. 1 and at different values of R, in the range $R = 0 \ldots 200\ \Omega$. (The feedback signal in the positive feedback loop is small at small R, and it is large at large R.) — The idealized system in Fig. 1 has the voltage transfer function[1] (choosing 10^{-6} sec as unit of time):

$$\frac{U_2}{U_1} = -\frac{1.5(2rs + 1)}{400\,rs^2 + (200 - r)\,s + 1} \tag{1}$$

[1] Expression (1) means the operational form of the unterminated output voltage (U_2) if the input voltage is the unit-impulse (i.e. $U_1 = 1$). Hence U_2 as function of time (if U_1 is the unit-impulse) is:

$$U_2(t) = \frac{1.5}{400\,r(z_2 - z_1)}\left[(2rz_1 + 1)\,e^{z_1 t} - (2rz_2 + 1)\,e^{z_2 t}\right]$$

where z_1 and z_2 are the zeros of $400\,rz^2 + (200 - r)\,z + 1$, a polynomial of the complex variable z. The value of z_1 and z_2 is characteristic of the type of equilibrium (see also discussion). So it is seen that form (1) reveals all about the equilibrium (the type of response) of the system.

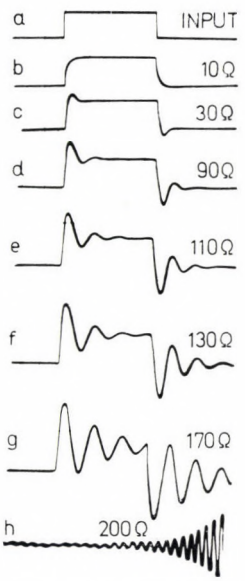

Fig. 2. Responses of a realization of system in Fig. 1 to a 9 ms, 50 mV rectangular voltage pulse. In the realized system the amplifiers consisted of transistors, resistors and batteries. *a*: input signal, *b*—*g*: output signals at the values from $R \sim 10$ to 170 Ω, *h* (slower beam): self-induced, diverging oscillation with no input signal in the unstable case, *R* slightly above 200 Ω

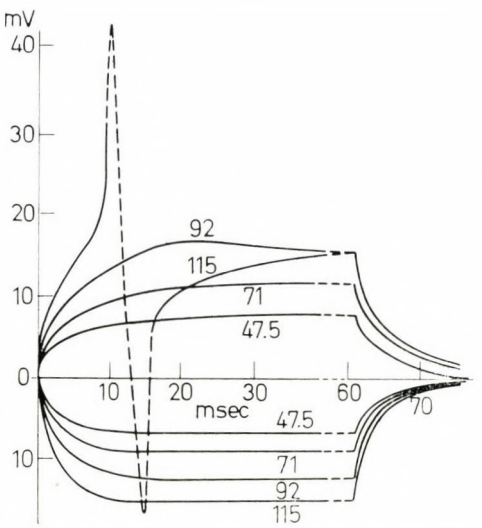

Fig. 3. (Katz, 1948.) Records of subthreshold potential changes in frog muscle fibres, when the stimulus is a long rectangular current pulse. Cathodic potentials shown as upward deflections. Numbers show current strength relative to rheobase

where r denotes the numerical value of R in ohms, s is the "operator of the differentiation", also called the "Laplacian" operator (Roberge, 1967). According to the calculation the response from 0 to about 20 Ω must be stable and non-oscillatory; from 20 Ω to 200 Ω stable and oscillatory; and above 200 Ω it turns unstable and the system is oscillating with no input signal; here the real system starts with "self-induced" linear oscillation and then it leaves the linear region.

2. The active elements (gain elements) of an active network as that of Fig. 1 may be negative resistances (realizable e.g. by tunnel diodes) or other controlled sources (e.g. ideal current amplifiers, realizable approximately by transistors). The realizability by solid-state electronic systems has a biophysical significance.

Fig. 4. (Hodgkin, 1948.) Similar record as those of Fig. 3, obtained in Carcinus axons

– The present experimental demonstration permits to raise the question whether passive or active electronic systems can produce the subthreshold linear responses observed in neural systems, e.g. the three types of responses mentioned in the introduction. Of these, the 1st type was recorded by Katz, Hodgkin and Knutsson in the cited and also in other works and attributed to a simple passive circuit. As illustrative examples we show the records of Katz and Hodgkin (Figs 3 and 4); one may compare these figures with Fig. 2b (Ernst, 1968).

Discussion

The neural responses in Figs 3 and 4 do not give real explanation, why the small-signal models need to be active (and do not give explanation for the second order model examined here). Based on physiological data, Roberge (1967) suggests a second order transfer function for the linear subthreshold responses of a neuron:

$$\frac{K(sT_1 + 1)}{(sT_2 + 1)(sT_3 + 1)} \tag{2}$$

where K is a scaling factor and T_1, T_2, T_3 are positive quantities. So it was natural to take such a simple expression as starting-point. But the 2nd and 3rd types of neural responses were also to be taken into account. Experimental records of the 2nd type are available in the literature (Arvanitaki, 1939, 1942; Hodgkin, Huxley, 1952; Hodgkin, Frankenhaeuser, published by Huxley, 1959 etc.), and this holds true also for the 3rd type (Arvanitaki, quoted papers). All the three types have been obtained also in theoretical models of excitation (Hodgkin, Huxley, 1952

and Huxley, 1959 by numerical calculation of responses; FitzHugh, 1961, Chandler et al., 1962, Hearon, 1964 and Lewis, 1965 by discussing the linearized system about the singular point). In the experimental and theoretical investigations referred to it is a single parameter (mostly the external Ca concentration) the varying of which can change the response from the 1st type into the 2nd, and from the 2nd into the 3rd type. So in view of the oscillatory and unstable linear neural responses it was also natural to generalize expression (2) so that after changing a single parameter in the network, T_2 and T_3 turned imaginary with positive real parts (stable oscillatory case), and after further changing this parameter the real parts became negative (unstable oscillatory case). And since a network can be unstable only if it contains gain elements, the amplifiers are necessary in the model. – For this reason we can suggest that the neural system operates even in the linear region as an *active electronic system*, or more precisely, as *an equivalent* of an appropriate active network. The question of what particular kinds of amplifiers could explain this activity (e.g. negative resistances or other controlled sources) would deserve some further exminations.

Acknowledgement

The author expresses his thanks to Professor E. Ernst who called his attention to this question. — Dr. J. Örkényi constructed the amplifier which has been employed in the system shown in Fig. 1.

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On the Role of Negative Resistance in Explaining Linear Neural Responses

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On the basis of the instability of the resting point it was assumed in a previous paper that the neural system, even in the linear region, is an equivalent of a network containing gain elements. This assumption is supported by the data making likely the presence of negative resistance in the nerve. In the present paper it is proposed that, besides the negative resistance, other active elements are required to explain the linear neural responses, even the oscillatory ones. The question how to transfer results to distributed models, thus interpreting them by electron processes, is also raised.

Introduction

The development of the neural periodicity (train of nerve impulses) is closely related to the instability of the resting point ("singular point"),[1] and hence to the instability of the linear (approximating) mathematical model of the system.[2] If the singular point turns unstable (e.g. spontaneously or owing to a constant stimulus) the system leaves the resting point and two possible cases may take place: 1. Either a stable limit cycle is formed and is occupied by the phase point (the point representing the state of the system), 2. or the system, after an impulse, returns to the resting point, then the instability comes into force and the process will be repeated. In both cases a train of nerve impulses, "repetitive firing" develops. Thus the question of the stability of the linear approximation is an important question in this respect (and it can be examined in a suitable small-signal model of the system; see Királyfalvi, 1968). The repetitive firing itself proves the possibility of an unstable equilibrium. Also direct experimental data reveal this possibility. Such events have been recorded to occur in the subthreshold linear region, corresponding to an unstable oscillatory equilibrium. Arvanitaki obtained stable and unstable oscillatory local responses by lowering the Ca concentration of the fluid bath and of the fibre ("decalcification") (Arvanitaki, 1939, 1942). In such

[1] Treating the neural system as a dynamic system, we can assume normally one real singular point. The known theoretical nerve models agree with this. For definitions see e.g. Minorsky, 1962.

[2] The linear approximation is meant in the neighbourhood of the singular point (in the subthreshold linear region).

an experiment the giant axon of Sepia officinalis was, for instance soaked locally in a mixture of 8 parts of sea-water and 2 parts of isotonic trisodium citrate solution, and it was examined as an electric two-port with input and output ports formed by four adjacent points of the axon surface (Arvanitaki, 1942). The stable oscillations in the decalcification experiments turn unstable with more intense treatment. Some experimental data allow also the assumption of the occurrence of non-oscillatory unstable equilibrium (Hodgkin, 1948). The stability of the singular point has been discussed theoretically in both the Hodgkin—Huxley model (Hearon, 1964; Chandler et al., 1962; Lewis, 1965) and the Bonhoeffer – van der Pol model (FitzHugh, 1961), with the result that it should be unstable under certain conditions. Huxley has calculated such cases in the model nerve when the very small local oscillations increase in amplitude until the system leaves the linear region and a spike starts. (This occurred in the case of lowering external [Ca] of the model. Huxley, 1959.)

Such a ("potentially unstable") system is necessarily active or more precisely, in terms of the network theory, it is equivalent to a network containing gain elements. (Clearly, a linear network is of interest here.) Concerning the gain elements, further requirements stem from the oscillatory linear responses. – We should like to consider what amplifiers may occur in a neural system, focusing attention on the negative resistance. This will be discussed in Section I in terms of networks (lumped equivalents) and then, in Section II, it will be studied the possibility of transferring results to distributed parameter systems, more real neural models.

I. Role of the negative resistance in the lumped parameter nerve equivalents

1. *Its occurrence in nerve fibres.* In the last few years, a number of experiments were reported, on the strength of which a steady-state negative resistance can be supposed to be present in the nerve fibre (Moore, 1959; Spyropoulos, 1965; Müller-Mohnssen, Balk, 1965; Balk, Müller-Mohnssen, 1966). In the experiments the negative resistance appeared in the measuring circuit after cooling, after application of veratridin, after increasing the external K-concentration, and also after other influences.

2. *Its amplificative nature.* Such an active one-port (a voltage or current source) is meant by an amplifier, whose voltage or current is controlled by another variable of the network (a voltage or current). For this reason the amplifier is synonymous with dependent or controlled source (Ghausi, 1965; Linvill, Gibbons, 1961). The active character of the amplifier means that, when connected to a passive network, the current flow always transmits power to the passive network (amplifier = gain element). The negative resistance itself possesses these properties. It is *controlled* since its voltage is proportional to another variable, i.e. to its own current. And it is *active*, i.e. it is a source of energy. When considered as a one-port, and connected with a network having Z driving-point complex impedance

with positive real part, the power reflection coefficient (Ghausi, 1965) is $\left|\frac{R+Z^*}{R-Z}\right|^2$ this value is always *greater than unity* (since $-R < 0$, and Z has a positive real part). (Z^* is the conjugate of Z.) Considering the $-R$ negative resistance as a two-port (Fig. 1e) the power gain can be examined with similar result. – For negative resistance as source of energy see Andronov et al., 1966.

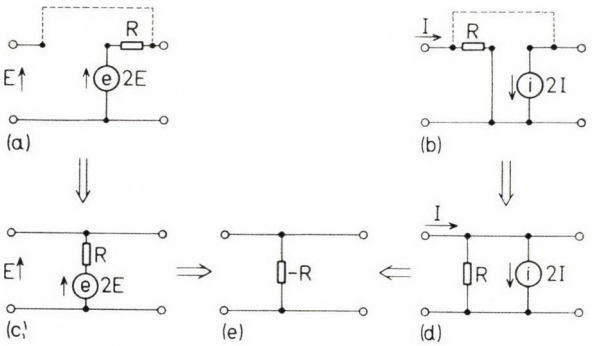

Fig. 1. Derivation of negative resistance from non-ideal voltage or current amplifier. (*a*), (*b* (without the dotted line): The original voltage resp. current amplifier, in symbolic represen tation. E and I denote input voltage and current, resp. (*c*), (*d*): The internal feedback ampli fiers obtained after shorting with the dotted lines. (*e*): The negative resistance equivalen to either (*c*) or (*d*)

As seen in Fig. 1 the $-R$ negative resistance arises from either an ideal voltage amplifier or an ideal current amplifier (having R output and input driving-point resistance, resp.) if we connect the output to the input. Thus the negative resistance can be considered as an internal feedback amplifier. This is seen from the two-port parameters of Fig. 1e. For instance $h_{12} = -g_{12} = 1 \neq 0$.

3. *It suffices to explain instability.* Networks (even *RC* networks) containing $-R$ as the only active element may be unstable. An unstable circuit arises e.g. by loading $-R$ with a capacitance. The same possibility in the oscillatory case is seen from the power gain or power reflection coefficient (see preceding paragraph) on the basis of their possibility to be infinite with proper load terminations.

4. *Can it explain also oscillatory responses?* If we assume resistance and capacitance as elementary passive parameters of the neural system, the linear equivalent will be an active *RC* network. Let us assume the active elements to be $-R$. It can be shown that an R, $-R$, C network *cannot have any oscillatory* network function. (This is shown in several works for positive R-s – e.g. Kuh, Pederson, 1959 –; the proof can be extended over arbitrary R-s.) Thus another active element should be assumed besides, or instead of, the negative resistance to explain oscillatory linear responses. This would also allow to reproduce the nerve inductance (Cole, 1941) by elementary processes.

II. Possibilities in the distributed parameter case

It should be emphasized that any network model is only an *equivalent* (substituting) network for the nerve fibre which (even the myelinated one) is in reality, a distributed parameter system. – Of course, networks are useful tools for several reasons: (*a*) Any given distributed system can be well approximated by them. (*b*) More simple mathematical treatment in many cases. (*c*) Demonstrativeness, the possibility of a graphic description. – Nevertheless, it is important that the results obtained by means of the network models be properly interpreted for the real nerve fibre. Two stages would be involved here: (1) Construction of a distributed model in accordance with the results of network-theory. (2) Physical interpretation of the parameters of the distributed model. – Our possibilities of accomplishing these stages can be considered for two types of distributed systems.

1st type, where the parameters can be considered to be distributed in one or two dimensions; such are the transmission lines and integrated solid state circuits. The structure of these systems is not quite homogeneous macroscopically since the boundary layers and thin films play an important part in them. Series (longitudinal) resistance, shunt or leakage conductance, capacitance between the layers, $p - n$ junction, etc. may be distributed.

2*nd type*, where the parameters are distributed in all three dimensions. The Gunn oscillator (Gunn, 1964) is a good example for this type. It consists of a crystal of *n*-type GaAs or InP homogeneous in structure macroscopically. In spite of the simple structure, it has an unstable oscillatory admittance at a definite biasing (about 3000 V/cm field at the GaAs).

Regarding the nerve membrane, the 1st type may take a great part in the nerve. Such a system turns up directly in physical terms as follows. One of the capacitances of the lumped model can be immediately interpreted as membrane capacitance. It is also possible to interpret the negative resistance, if we assume $p - n$ junction in the membrane or a thin layer of dielectrics which may effect a "tunneltron" type negative resistance (for tunneltron see Gentile, 1965). We refer to the experimental data mentioned in paragraph I/1, as well as to Wey, who has revealed the possibility of electron tunneling through a nerve membrane (Wey, 1967). In order to model the nerve impulse Scott designed a transmission line loaded with an idealized, distributed tunnel diode (negative resistance), referred to as a line of "neuristor type" (Scott, 1963). Challice and Fry reported the employment of integrated circuits in neural analogs (Challice, Fry, 1966). – However, when interpreting the negative resistance through the membrane, we have to remember that *solely*, the membrane is insufficient for this. At the tunnel diode or the tunneltron, there is a solid state structure in contact with the boundary layer supplying the electrons or holes.

As to our above conclusion concerning the occurrence of other active elements, its interpretation in the distributed (1, 2, or 3 dimensional) case is a task for the future. Distributed controlled sources e.g. in the nerve axoplasm are conceivable in several forms (see below). The highly complex molecular structure of

the axoplasm should be remembered here (Schmitt, 1959; Ernst, 1968). In addition, in the case of the heart muscle, Challice and Fry suggested that the transmission of the sarcoplasmic reticulum was an active rather than a passive phenomenon (Challice, Fry, 1966).

When emphasizing the role of the axoplasm's structure we imply that a distributed system of *the 2nd type* should take a similarly important part in the electrical activity of the axon. Such a system permits the development of a solid-

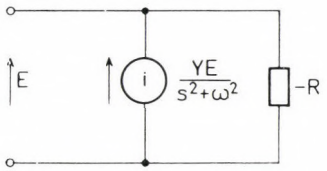

Fig. 2. An equivalent for the Gunn oscillator similar to that proposed by Gunn. — R represents a negative resistance. Symbol i inside the circle represents a current source whose current equals $\frac{YE}{s^2 + \omega^2}$, a function of E. E is the input voltage, s is the operator of differentiation, Y and ω are appropriate constants

state excitation theory (interpretation by electron and quantum processes), a modern approach of excitation (Ernst, 1966; Ernst et al., 1967). So the presence of some *bulk effect* (besides the surface effects) would be supposed. This assumption would gain a strong theoretical support if it succeeded in reconstructing the lumped nerve models in a 2nd type, bulky distributed form. There are facts indicating that such a reconstruction may be possible. — 1. The Gunn effect is an example that a "homogeneous" substance may exhibit a rather complicated type of driving-point or transfer functions, due to the energy-band structure of the substance. 2. The positive and negative resistances are originally bulk properties (resistivity!), hence they can be interpreted immediately. 3. Other types of amplification may also be interpreted in the 3 dimensional case. As an example the molecular amplification by the MASER-effect should be mentioned (Altschuler, Kosyrew, 1963). Gunn proposed for the Gunn oscillator an equivalent circuit as follows: "a parallel combination of a frequency-independent negative resistance, shunted by a constant-current AC generator . . . whose amplitude is a function of applied voltage" (Gunn, 1964). Such a circuit is shown in Fig. 2. This contains a particular kind of transadmittance amplifier. Consequently, such an amplifier would be distributed in the Gunn oscillator.

The capacitance occurring in the lumped model can be interpreted in this case only as *equivalent* capacitance which arises from the internal electron and molecular processes. At any rate, energy storage processes (such as those effected by capacitance or inductance)[3] *are requisite for the oscillations*, but these can arise,

[3] Or in more general, delay effects.

in all certainty, from the interaction of the electrical and non-electrical processes of homogeneous material. – "Time of relaxation of concentration profiles of injected carriers and related transport effects in solid state p-n elements ... can give rise to timevariant resistance properties" — i. e., to energy storage properties (Mauro, 1961). For some further details see Linvill, and Gibbons, 1961. As another example the piezoelectric crystal may be mentioned which produces equivalent capacitance as well as inductance due to the interaction of mechanical and electrical events.

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Book Reviews

Advances in Enzyme Regulation, Volume 5. Edited by GEORGE WEBER. Pergamon Press, 1967, 469 pages

The volumes of Advances in Enzyme Regulation contain the papers presented at the annual Symposia at Indianapolis, USA (Proceedings of the Symposium on Regulation of Enzyme Activity and Synthesis in Normal and Neoplastic Tissues). The fifth volume is devoted to the 1966 Symposium.

Like the preceding four volumes this fifth volume, too, deals with a broad field of enzyme regulation. The authors present first a valuable short survey of their subject and then describe their own, partly already published, partly new experimental material. The applied methods are quoted in a relatively brief, concise form.

One of the valuable features of this series is the yearly regular appearance of its volumes. This enables the reader to follow with ease the development and more important results in the various branches of this field of research. Publication of the discussion after the sessions of the various sections presents an interesting complementation to the papers. Unfortunately, in the course of years these discussions have been gradually abridged till they have become more supplementary contributions than true discussions.

Each paper is followed by a detailed list of references. The authors' and subjects' indexes at the end of the book facilitate its use.

In the Fifth Volume Chance's paper in Session I (Enzyme Regulation and Coenzyme Metabolism) is of particular interest as it demonstrates the maleate dehydrogenase function of ascitic cells *in vivo* and the changeability of the redox state of cytosol.

The subjects of the papers in Sessions II and III (Enzyme Regulation in Different Organs, in Kidney) were: the DNA-polymerase activity of the prostate under the action of testosterone; the role of RNA and protein synthesis in the lipolysis of isolated lipid cells; the organ specific control of glutamine metabolism and the role of glutaminase isozymes in the latter. These few quotations may be sufficient to show the rather great variation of subjects in the different sessions and certain overlappings due to the time schedule of the Symposium. For the sake of simplicity of the other Sessions only the titles should be mentioned.

Session IV: Metabolic Regulation Through Enzyme Biosynthesis
Session V: Role of Enzymes in Development
Session VI: Comparison of Enzyme Regulation in Mammalian and Microbial Organisms
Session VII: Gluconeogenesis
Session VIII: Hormone Action *in vitro*
Session IX: Regulation and Isozymes
Session X: Regulation in Cancer
Session XI: Special Symposium Lectures (The Redox State of NAD in the Cytoplasm and Mitochondria)

Even a brief description of each paper would be far beyond the scope of this review. The papers include the very wide field of enzymology as well as topics which are also implicit in the *in vivo* regulation of metabolic processes. Some of the papers are of a more general nature and provide certain summaries, while others deal only with some details. Compared to the papers published in the pertaining journals the communications of the Symposium contain rather little that is new, but offer instead a very good possibility of a general survey of certain topics especially with respect to the *in vivo* estimation of enzymatic processes. The volume has the further advantage of publishing the work of several authors on

the same subject, thereby offering a means of comparison.

The program in Volume V gives a brief survey of the contents of the preceding volumes. Thus Volume I deals with the enzymatic regulation of a single organ, namely of the liver, including biochemical, physiological and pathological problems.

Volume II is also devoted mainly to the enzymes of the liver with special emphasis on the regulation of gluconeogenesis, and the effect of feedback regulation on the activity and synthesis of hepatic enzymes.

Volume III deals not only with the enzymatic regulation of the liver, but also of the muscle, the development of enzymes and the problems of hepatoma regulation.

Volume IV contains a review of the regulation of lipoid metabolism and several papers on enzyme induction and on the role of adaptation regulation in metabolism.

GY. BOT

Callus Formation Symposium on the Biology of Fracture Healing. In English. Editors, I. KROMPECHER, and E. KERNER. Publishing House of the Hungarian Academy of Sciences. Budapest 1967.

The book contains the papers presented at the Symposium held in Debrecen in July, 1965, at which 55 Hungarian scientists and 27 researchers from abroad participated. The Symposium was organized by Prof. István Krompecher, Head of the Department of Anatomy, Histology and Embryology of the Debrecen University of Medicine. Professor Krompecher and his team have been engaged for several decades in the study of the mechanism and biology of the healing of bone fractures and of the biochemical processes which accompany callus formation. As a result of their experiments they came to the conclusion that the formation of the cartilaginous tissue takes place in the so-called avascular medium which is very poorly provided with capillaries and blood vessels. As a consequence due to the lack of oxygen the formation of the cartilage in the regenerating granulation tissue is associated with the glycolytic processes of carbohydrate breakdown. As a result mucopolysaccharide formation is enhanced and from this the basic substance of the cartilaginous tissue is built up. It appears that during the development of chondrification of the enzymes in the Embden—Meyerhof scheme the partial inhibition of phosphofructokinase leads to a change in the direction of metabolism. In this course the glucose-6-phosphate and fructose-6-phosphate are converted to hexosamine precursor and this latter polymerizes with the uronic acids forming thereby chondroitine sulphates, hyaluronic acids and keratosulphates. These last substances participate in the building of the substance of the callus.

The work of Prof. Krompecher and his team extended in addition to the study of the above-outlined biochemical processes also to the morphology, histology, histochemistry of callus formation including the physical factors involved. Their work was met with great interest not only of theoretical research workers but also of clinicians. This explains the presence of several surgeons from abroad (Böhler, Küntscher, Ostrowski, Petrokov, Schenk, Vukelic, Willenegger) at the Symposium. These authors presented papers on the clinical aspects of bone fractures which they illustrated with many photographs and drawings.

The most up-to-date trends in the study of callus formation were presented by several well known experts of the field.

The topography and development of osteogenesis after fracture is reflected in the electron microscopic pictures. It has been found that the mature osteoblasts synthesize the two components of the osseous tissue, namely the mucopolysaccharides and the scleroproteins neither at the same location nor at the same time, though the two types of substances will leave the mature osteoblasts together and will form concomitantly the intercellular substance of the osseous tissue.

Autoradiographic and radiochemical investigations gained in importance from the aspect of histopathological localization, especially as the sulphur isotope indicates the regeneration of mucopolysaccharides, while proline labelled with tritium provides information on the formation of collagen. When coupled with experiments on tissue respiration these methods proved to be

suitable for the study of the effect of cortisone and oxyphenobutazone.

Two important papers deal with the structure and metabolism of the cartilage and bone from a biochemical aspect; these papers include a valuable list of references and sum up the achievements of the last ten years in this field.

There are further very interesting papers among the 62 in the book, particularly on details which give for the experts a valuable survey on the various trends in callus research. Unfortunately the great variety of subjects prevents the reviewer to deal with every detail.

In fact the material presented in the volume is highly manifold, perhaps because many Hungarian investigators are engaged in the study of this problem. They have summed up the best part of their achievements and results, so that the reader will be able to ascertain their contribution to the growing problem of callus formation by their many valuable observations and the initiation of new research trends. In his preface Prof. Krompecher points out the growing number of accidents involving bone fractures due to industrialization and the rapid development of the means of transport as a result of which the problem of the healing of fractures, the study of callus formation, has become a matter of great interest.

The book lacks an authors' and subjects' index.

I. B. BANGA

Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója Műszaki szerkesztő: Farkas Sándor

A kézirat nyomdába érkezett: 1968. VII. 18. — Terjedelem: 9,75 (A 5) iv, 79 ábra

68,65979 Akadémiai Nyomda, Budapest—Felelős vezető: Bernát György

Biosynthese der Alkaloide

Edited by Prof. Dr. K. Mothes — Prof. Dr. H. R. Schütte

1968, about 750 pp., size 165×230 mm, leatherette, ca. 135.— Mark DDR

Forthcoming autumn 1968 in German language

The early publications on the chemical nature of substances synthesized by living organisms opened the discussion on the mechanisms of their formation. The alkaloids have attained particular importance due to their physiological and pharmacological effectiveness. Investigations in this field, however, remained almost exclusively speculative up to the 'fifties'. It was not until radioactive isotopes were applied about 15 years ago that great advances towards forming exact conceptions could be made. The results on the biosynthesis of alkaloids obtained in many laboratories all over the world have been reviewed critically and a comprehensive presentation has been made in the present volume by scientists having ample experience in this field. The number of contributors has been kept small and they are all members of the same team, which makes for a coherent presentation. Special care has been taken to consider the reaction mechanisms in interpreting the various findings. Much space has been devoted to the chapters that deal with the biology and genetics of alkaloid formation, which are often neglected in publications of a purely chemical character. Since alkaloids chiefly originate from the amino acid metabolism the biosynthesis of the amino acids involved in the formation of alkaloids has also been summarized. A detailed discussion of the known methylation and transamination reactions is included with particular emphasis placed on their importance for alkaloid biosynthesis.

Considering that today the biosynthesis of alkaloids is an imposing, almost complete system this book is to be recommended not only to research workers in chemistry, biology, biochemistry, pharmaceutics, the medical sciences and agriculture who are concerned with alkaloids, but it will no doubt prove useful and inspiring to all those who take a general interest in the ways living nature, in an almost unlimited variation of simple principles, creates extremely simple as well as highly complex entities, using only a small number of reaction types and substances of the intermediary metabolism.

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Radioaktive Isotope in der organischen Chemie und Biochemie

(Radioactive Isotopes in organic chemistry and biochemistry)

by H. R. SCHÜTTE

273 pages text, 416 pages tables, 52 illustrations
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This book ensued from lectures, which for some years the author has been giving for students of organic chemistry and biochemistry at the Martin Luther University Halle-Wittenberg, GDR. According to its contents it is meant for the large circle of people who work with radioactive isotopes in organic chemistry and biochemistry and in the related branches of medicine, pharmacology and agriculture. But only tracer application is dealt with; fields, in which radiation is an end in itself are not treated.

In his book the author especially goes into the problems and application of the low-level β-radiators carbon 14 and tritium. In an introductory chapter the reader's mind is refreshed on the general principles of atomic structure, of isotopy and of radioactivity in a condensed form. The preparation of labelled compounds is dealt with exhaustively. The respective chapter contains directions on syntheses with labelled substances and a number of standard directions as well as preparative-synthetical methods and biosynthetical and radiochemical processes for preparing labelled compounds. In a supplement (stored knowledge!), most of the syntheses of labelled organic compounds known until the summer of 1965 are summarized in tables. The literature for this supplement was evaluated until March 1966. *The extensive section of tables makes this work a valuable and significant source also for readers who are not familiar with the German language.* Modern biochemistry is unthinkable without isotope technology. This book was written for the purpose of introducing the reader to this indispensable resource of modern research and of facilitating the specialist's search of proved methods.

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Regulation of the Activities of Various Aminoacyl-tRNA Synthetases by Testosterone in the Seminal Vesicle of the Rat

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(Received March 26, 1968)

The activities of leucine, isoleucine, lysine, methionine and valine activating enzymes of the seminal vesicles of normal and castrated rats as well as of testosterone-treated castrates were assayed in the 105 000 *g* supernates of vesicular homogenates, on the basis of their ability to stimulate the amino acid dependent ATP—PP exchange reaction.

The rate of ATP—PP exchange catalyzed by vesicular activating enzymes is fundamentally influenced by the Mg^{2+} ion concentration of the incubation medium. High level of mercaptoethanol in the homogenizing medium and in the incubation mixtures has a promoting effect on the exchange rate.

Our results indicate that testosterone regulates the levels of these activating enzymes in the vesicular tissues and activation or inhibition of the enzyme activities may not play any role in the regulation. Following castration the decrease in the activities is exponential with a half time of about 1.5 days. In response to testosterone administration the foregoing amino acid activating enzymes of the castrated animals begin to accumulate progressively after a lag period of about 12 hours. The rate of accumulation of the various activating enzymes seems to be different. The activity of the leucine activating enzyme is enhanced most rapidly, whereas the activity of the valine activating enzyme increases at the smallest rate. The increase in the activity of leucine activating enzyme is about three times as rapid as that of the valine activating enzyme and 4—5 times more rapid than the rate of accumulation of the "soluble proteins" of the vesicles.

Introduction

Evidence accumulated during the past few years disclosed that the regulation of protein synthesis in the androgen dependent organs is mediated by the primary action of testosterone on the RNA metabolism (Williams-Ashman et al.,

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Abbreviations used:

ATP	— Adenosine 5'triphosphate	DNA	— Deoxyribonucleic acid
PP	— Pyrophosphate	TCA	— Trichloro-acetic acid
RNA	— Ribonucleic acid	EDTA	— Ethylene-diamine-tetraacetate

1964; Williams-Ashman, 1965; Kenney, 1965; Liao, 1965; Liao et al., 1965, 1966, 1966a; Liao, Lin, 1967). However, little attention has been paid to the investigation of the effect of testosterone on the rate of synthesis of specific enzyme molecules. In the course of a work on the regulation of protein synthesis in the seminal vesicle of the rat we became interested in the effect of testosterone on the activities of the enzymes considered at present indispensable for protein synthesis. Among the androgen dependent organs the seminal vesicles seemed most preferable for the experiments. As it has been shown, the main function of this gland is to produce a secretion rich in a clottable, strongly basic protein as well as in other proteins (Mányai, 1964a). Although the synthesis of the secretory proteins has proved to be very sensitive to testosterone, as a response to this hormone the rate of labeling of the bulk proteins of the seminal vesicle increases first, preceding the greater increase in the rate of labeling of the secretory proteins (Tóth, Mányai, 1968). We inferred from our latter result that the synthesis of some proteins, presumably enzymes, are especially important for the functioning of the gland. In view of their obvious importance in the function of the seminal vesicle, studies on the regulation of activities of the amino acid activating enzymes (Amino acid-RNA ligases, E. C. 6.1.1.) and of the aminoacyl "transfer" enzymes have recently been carried out in our laboratory. Kochakian et al. have found previously that the androgen state of the animals has a significant influence on the activities of amino acid activating enzymes in the seminal vesicles of the guinea pig and in the kidneys of the mouse (Kochakian et al., 1961, 1963). In the experiments presented here the role of testosterone in the regulation of the levels of various amino acid activating enzymes in tissues of the rat vesicles will be documented. Studies on the regulation of "transfer" enzyme activity by testosterone will be published elsewhere.

A preliminary account of these results was given earlier (Tóth, Mányai, 1966; Tóth, Machovich, 1967).

Materials and Methods

Male rats from the inbred strain of the National Fodor Sanatorium were used in the experiments. All the animals were fed on a normal laboratory diet and received water ad libitum. Castration of adult rats with 250–350 g body weight was performed in ether anaesthesia via the scrotal route. Whenever the effect of testosterone was studied, castrated rats were given 5 mg of testosterone phenylpropionate (Retandrol, Richter) in the form of intraperitoneal injection at different times before killing. If the testosterone effect was investigated for periods longer than two days the Retandrol injections were repeated daily.

The animals were killed by decapitation, the seminal vesicles were removed and immediately placed in ice cold homogenizing medium containing sucrose (0.25 M), mercaptoethanol (0.001 M), EDTA (0.001 M) and Tris-HCl buffer of pH 7.6 (0.02 M). The pooled seminal vesicles of a respective group of animals

were freed from the coagulating gland and adhering secretory fluid. The glands were then blotted, weighed, chopped with scissors and homogenized in most cases with 20 volumes of the homogenizing medium in a Potter-Elvehjem all glass tissue homogenizer. All these manipulations were carried out near 0 °C. The 105 000 *g* supernates of the homogenates served as enzyme preparations.

Amino acid activating enzymes were assayed on the basis of their ability to stimulate the amino acid dependent ATP-pyrophosphate exchange reaction (Hoagland, 1955). The reaction mixtures contained 5 μmoles of ATP, 3–5 μmoles of ^{32}PP, 4–20 μmoles of $MgCl_2$, 9 μmoles of NaF, 2–6 μmoles of L-amino acid, 100 μmoles of Tris-HCl (pH 7.6) and 100–200 μg of supernatant protein in a final volume of one ml (amounts of the components and further details are described in the protocols). Incubations were carried out at 37 °C for twenty minutes. The reaction was stopped with 1 ml of 20 per cent TCA and ATP was extracted by a procedure similar to that of Crane and Lipmann (1953). The enzyme activity was expressed, unless otherwise stated, as μmole PP exchanged per hour/mg supernatant protein. Figures were corrected by subtracting the value of the ATP–PP exchange obtained without added amino acid (background exchange).

Measurement of ^{32}P radioactivity was carried out on glass planchets, using a thin mica end-window G. M. tube, or utilizing the Cerenkov radiation (von Haberer, 1965) by means of a Packard Liquid Scintillation Spectrometer.

Technical details of the in vitro labeling of vesicular proteins with ^{14}C-valine and the preparation of samples for the determination of radioactivity were essentially the same as previously described (Mányai, 1964a), except that hot saline and hot acid washings were omitted from the procedure. The ether dried powder of the washed acid precipitate was layered on alumina planchets and measured for radioactivity using an end-window G. M. tube. Further details are given in the legend to Fig. 4.

Phosphate determinations were made by a modified method of Fiske and Subbarow (1925) using ascorbic acid as reducing agent.

DNA was determined from tissue portions pooled for this purpose and homogenized in cold perchloric acid. Separation of RNA and DNA was accomplished by the procedure of Schmidt and Tannhauser (cf. Leslie 1955), and the DNA content was estimated according to Dische (1955).

Protein contents were measured with the Lowry method (Lowry et al., 1951), horse serum albumin serving as standard.

Radioactive pyrophosphate was prepared by high temperature fusion of $Na_2H^{32}PO_4$ (Peng, 1956).

$Na_2H^{32}PO_4$ and (1–^{14}C) D,L-valine (specific activity: 9.89 mCi/mmole) used in the experiments were the products of the Isotope Institute, Budapest. Mercaptoethanol was obtained from Fluka, Switzerland. Crystalline ATP-Na and other chemicals were purchased from Reanal, Budapest.

Results

Catalysis of ATP–PP exchange reaction by aminoacyl-tRNA synthetases of the seminal vesicle

Throughout the experiments presented in this paper the freshly prepared and subsequently diluted 105 000 *g* supernate of the vesicular homogenate was used as enzyme preparation. When incubation mixtures contained supernatant protein up to 200 μg the rate of the ATP–PP exchange was a linear function of the

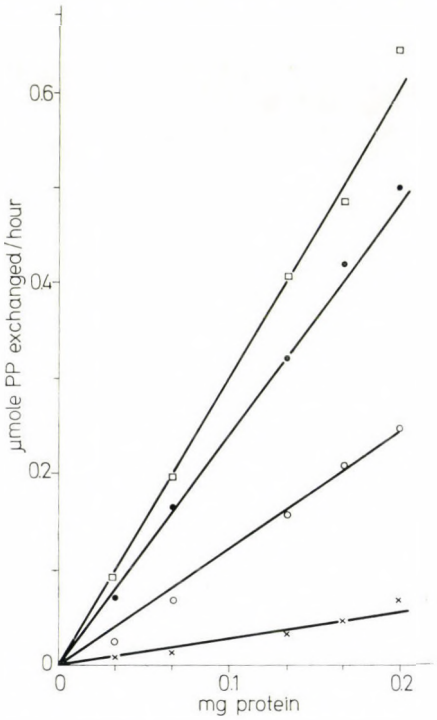

Fig. 1. Dependence of ATP—PP exchange rate on the concentration of supernatant protein. The effect of different Mg^{2+} concentrations and of mercaptoethanol

Enzyme preparation was made from the seminal vesicles of 4 normal rats as described in Methods. The 105 000 *g* supernate was diluted with 0.01 M Tris—HCl buffer of pH 7.6, except when mercaptoethanol was also included: here buffered mercaptoethanol (0.25 M) served as diluent. Incubation mixtures contained ATP 5 μmoles, ^{32}PP 5 μmoles, NaF 9 μmoles, Tris—HCl of pH 7.6 100 μmoles, supernatant protein as indicated on the diagram, plus

— × — × — 8 μmoles of $MgCl_2$
— ○ — ○ — 4 μmoles of $MgCl_2$, 1 μmole of each L-amino acid
— ● — ● — 8 μmoles of $MgCl_2$, 1 μmole of each L-amino acid
— □ — □ — 8 μmoles of $MgCl_2$, 1 μmole of each L-amino acid, 75 μmoles of mercaptoethanol

in a final volume of 1 ml

amount of protein added whether a mixture of amino acids or the individual amino acids were incubated with the supernate.

It has been shown by McCorquodale and Mueller (1958) that the rate of ATP–PP exchange is fundamentally influenced by the presence of reducing agents. Free-SH in the medium not only activates but also protects the activating enzymes from inactivation (Bublitz, 1966). Furthermore, different activating enzymes have been found functioning optimally at different concentrations of Mg^{2+} ions in the incubation media (Hele, 1961, 1964). In this respect the special importance

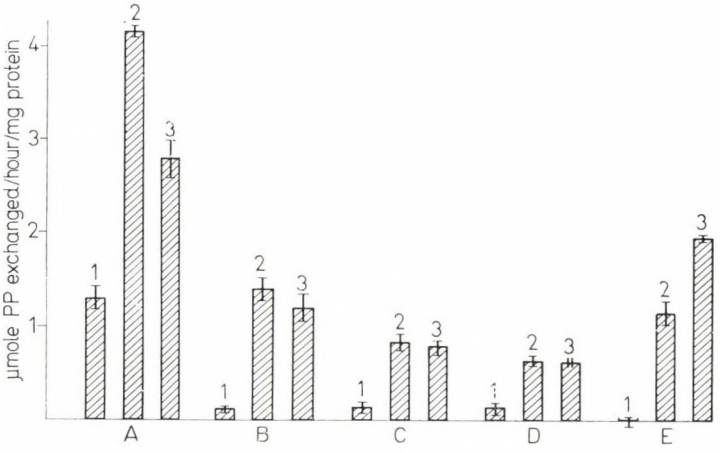

Fig. 2. The rate of ATP–PP exchange stimulated by the vesicular supernate in the presence of various mixtures of amino acids

The data were obtained from experiments in which the following groups of amino acids (2 µmoles of each amino acid) were incubated with the enzyme preparation:

A: leucine, isoleucine, methionine, valine
B: glycine, serine, alanine, threonine, cysteine
C: tryptophan, tyrosine, phenylalanine
D: glutamic acid, aspartic acid, proline, glutamine, asparagine
E: lysine, arginine, histidine

In the experiment the results of which are represented in columns N° 1, the supernate was prepared from the seminal vesicles of 4 normal animals as described in Methods. Aliquots of the diluted (0.01 M Tris–HCl) supernate containing 146 µg protein were incubated with ^{32}PP (5 µmoles), ATP (5 µmoles), $MgCl_2$ (4 µmoles), NaF (9 µmoles), amino acids as indicated above, Tris–HCl of pH 7.6 (100 µmoles), in a volume of 1 ml. Background exchange: 0.13 µmole PP/hour/mg protein.

The results of another experiments are represented in the columns marked with 2 and 3. The seminal vesicles of 5 normal rats were homogenized in a medium containing 0.01 M Tris–HCl, pH 7.6 and 0.25 M mercaptoethanol. Before incubation the supernate was diluted with 0.01 M Tris–HCl and aliquots containing 90 µg protein were incubated with ^{32}PP (3 µmoles), $MgCl_2$ (8 µmoles in series 2 and 20 µmoles in series 3), mercaptoethanol (15 µmoles) and amino acids, ATP, Tris–HCl as indicated above, in a volume of 1 ml. Background exchange: 0.4 and 0.37 µmole PP/hour/mg protein in series 2 and 3, respectively.

Incubations were carried out in duplicates, the values of the parallels are shown as brackets on the top of the columns

of measuring at optimum Mg^{2+}/ATP ratios to achieve optimal enzyme activities has been emphasized (Novelli, 1967).

In our experiments with the vesicular enzymes the rate of exchange with an equimolar mixture of twenty amino acids was doubled when Mg^{2+} concentration in the reaction mixtures was raised from 0.004 M to 0.008 M (Fig. 1). Storage of the enzymes in 0.25 M buffered mercaptoethanol at 0 °C for two hours, following an incubation in 0.075 M mercaptoethanol resulted in a further increase in the exchange rate (Fig. 1).

Stimulation of the ATP–PP exchange rate by the vesicular supernate was also tested with five arbitrary groups of amino acids (Fig. 2). At 0.004 M Mg^{2+} significant stimulation of the exchange reaction was measured only with the mixture of leucine, isoleucine, methionine and valine. However, when the enzyme activities were measured at 0.008 M, or at 0.02 M Mg^{2+}, with enzymes prepared in 0.25 M mercaptoethanol, the ATP–PP exchange rate was markedly stimulated with each group of amino acids as substrate. We found that similarly to the liver enzymes (Hele, 1961) the vesicular supernate could stimulate the lysine dependent ATP–PP exchange only when the Mg^{2+} concentration in the medium was higher than 0.01 M.

When about 200 μg of the supernatant protein or less was used for the assay of activating enzymes, the ATP–PP exchange was directly proportional to the incubation time at least up to 30 min. Hydrolysis of ATP or PP during the incubation was not appreciable even in the absence of NaF.

The additivity of the leucine, isoleucine, methionine, and valine dependent exchange reactions were also studied. The only case when additivity was not found occurred with the exchange reactions dependent on leucine and isoleucine.

The observations described above were made with the vesicular supernates of normal animals, however, they were found valid whether castrated animals or testosterone treated castrates were used for the experiments.

The differential increase in the activities of various aminoacyl-activating enzymes following testosterone administration

In this series of experiments the effect of castration and of testosterone administration to castrated animals, on the activities of leucine, isoleucine, methionine and valine activating enzymes were studied. These experiments involve the assumption that each of the amino acid dependent ATP–PP exchanges measured is catalyzed by a single aminoacyl activating enzyme.

Results of two similar experiments are summarized in Fig. 3. Adult normal rats were castrated and used for the experiments on the 17th day following operation. Testosterone treatment of different groups of the animals began at different times before killing, as indicated in the diagram. Enzyme preparation from intact litter mates of the castrated rats was also made in the experiment. The activating enzymes were determined as described in Methods.

Castration resulted in a decrease in the ability of the vesicular supernate to stimulate the ATP–PP exchange reaction dependent on either of the four amino acids. The extent of this decrease as compared to the corresponding normal value was in the range of 70–75 per cent. Testosterone treatment for different periods

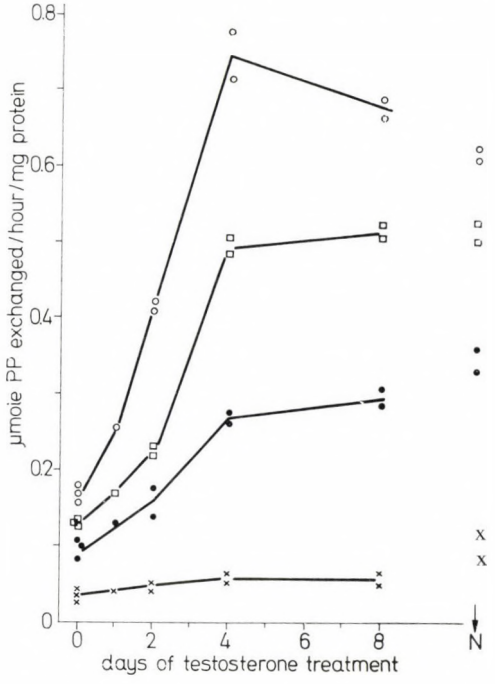

Fig. 3. Increase in the activities of leucine, isoleucine, methionine and valine activating enzymes induced by testosterone

In two experiments altogether 41 rats were castrated and sacrificed 17 days later. Different groups of castrated rats were treated with testosterone for various time periods before killing, as indicated on the abscissa. The seminal vesicles of 4—7 animals were homogenized and 105 000 *g* supernates prepared as described in Methods. Enzymes were prepared also from 4 normal litter mates of the castrates. Supernates were diluted with Tris–HCl and aliquots containing 150 to 200 μg protein were incubated with ATP (5 μmoles), ^{32}PP (5 μmoles), $MgCl_2$ (4 μmoles), NaF (9 μmoles), Tris–HCl (100 μmoles), 2 μmoles of the respective amino acid, in a volume of 1 ml.

Values of the background exchange were in the range of 0.05—0.15 μmole PP/hour/mg protein.

Each point represents the result of an incubation with one of the following amino acids: leucine (o), isoleucine (□), methionine (●), valine (×).

The activity of enzymes from normal, intact animals is marked with N on the abscissa

before killing resulted in a marked increase in the enzyme activities catalyzing the leucine, isoleucine and methionine dependent ATP–PP exchange reactions. The increase in activity of the valine activating enzyme exceeded only slightly

the increase in the supernatant protein. The pattern of time courses of the rise of various enzyme activities tested were similar to each other. However, substantial differences were noted in the enhancement of the various activities. On the fourth day of hormone effect the ATP—PP exchange rates were stimulated by

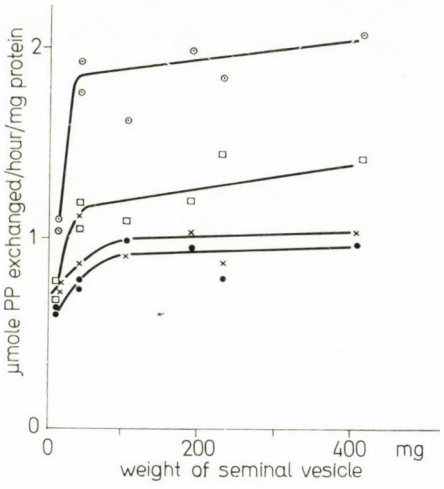

Fig. 4. Changes in the ability of vesicular supernates to stimulate the leucine, isoleucine methionine and valine dependent ATP—PP exchange reactions as a function of the wet weight of the seminal vesicles.

Enzyme preparations gained from the seminal vesicles of 40 immature rats and from different groups of 35 sexually mature animals were diluted with 0.01 M Tris—HCl and used for activity determination in the ATP—PP exchange test. Incubation mixtures contained: $MgCl_2$ (8 μmoles), L-amino acid (4 μmoles), protein (100—200 μg) and other components as indicated in the legend to Fig. 3, in a volume of 1 ml.

Values of background exchange were in the range of 0.4—0.5 μmole PP/hour/mg protein.

Each point represents the mean value of duplicate incubations with the same enzyme preparation. Different points symbolize the different amino acids added to the incubation media: leucine (o), isoleucine (□), methionine (●) and valine (×)

the vesicular supernate with leucine, isoleucine, methionine or valine as substrates 4.4, 3.8, 2.8 and 1.5 times more rapidly, respectively, than by the enzymes of untreated castrates. These differences are also reflected in the slopes of the curves. The exchange rate with leucine on the fourth day was even higher than the normal value, while the activities of the enzymes which activate methionine or valine, did not reach the normal levels even after as long a period as eight days.

It is known that the development of the seminal vesicles is regulated by testosterone. Therefore it was of interest to see whether a similar difference as above could be found in the case of the amino acid activating enzymes of the developing seminal vesicles. To test this idea enzyme preparations were made from

the seminal vesicles of immature, juvenile and adult rats, respectively. The activities of the activating enzymes catalyzing the leucine, isoleucine, methionine and valine dependent exchange reactions were plotted against the weight of the seminal vesicles (Fig. 4). From these data it became evident that "puberty"

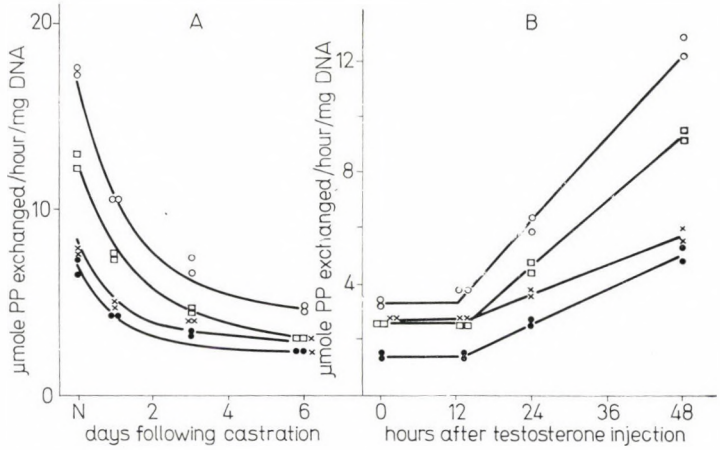

Fig. 5. Activities of leucine, isoleucine, methionine and valine activating enzymes in the early phase following castration and testosterone injection, respectively

Three groups of rats (at least 5 per group) were castrated in experiment A, at different times before killing as indicated on the abscissa of diagram A. The 105 000 *g* supernates were prepared from the seminal vesicles of castrates as well as from the glands of 4 normal animals. The activity of supernates to stimulate the ATP—PP exchange reaction was tested with four different amino acids as substrates.

This activity, tested separately with the above amino acids was also measured with enzyme preparations obtained from the seminal vesicles of castrated rats (at least 3 per group) injected with testosterone at different times before killing, as indicated on the abscissa of diagram B.

The composition of the incubation mixtures was exactly the same as in the experiments shown in Fig. 4. The specific exchange rates, expressed as μmole PP/hour/mg DNA were corrected for the background exchange (ranging from 1 to 4 μmole PP/hour/mg DNA) and indicated on the ordinates of the diagrams.

Each point represents the result of an incubation with one of the following amino acids: leucine (○), isoleucine (□), methionine (●) or valine (×)

caused the differential elevation of these enzyme activities in the vesicular supernate very similarly to that observed after testosterone administration to castrated rats.

The time course of the decrease of activities of various amino acid activating enzymes following castration and the induction of these enzyme activities after a single injection of testosterone were studied. The purpose of these experiments was to analyze the effect of testosterone on the activating enzymes of the seminal vesicles in detail during the early phase of hormone action. Enzyme activities were

referred here to 1 mg of DNA of the original homogenates the high speed supernates of which served as enzyme preparation. By the expression of enzyme activities in this way we expected to accomplish the best approximation of the changes occurring at the cellular level. The DNA content of the seminal gland was shown

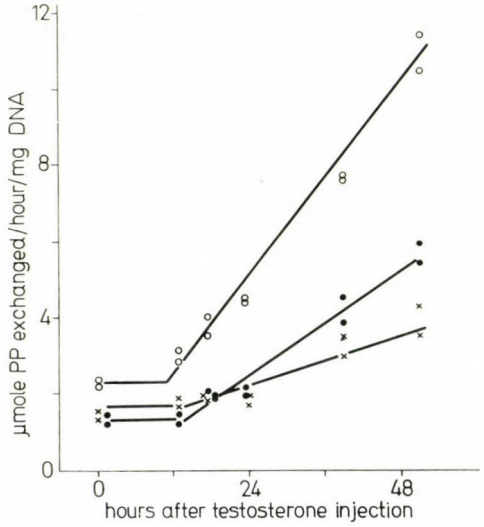

Fig. 6. Activities of leucine, lysine and valine activating enzymes in the early phase following testosterone injection

24 rats used for the experiment, 21 days following castration, were distributed into six equal groups, and the members in five groups were injected with testosterone at different times before killing, as indicated on the abscissa of the diagram. The 105 000 *g* supernates prepared from the vesicles were diluted with a solution of Tris—HCl (0.01 M) and mercaptoethanol (0.175 M). Aliquots containing 100—200 μg protein were incubated with 6 μmoles of ^{32}PP, 5 μmoles of leucine or valine, 8 μmoles of $MgCl_2$, 70 μmoles of mercaptoethanol and ATP, NaF and Tris—HCl as described in Methods, in a volume of 1 ml. Reaction mixtures for lysine dependent exchange contained 90—160 μg supernatant protein, 6 μmoles of lysine, 3 μmoles of ^{32}PP, 20 μmoles of $MgCl_2$, 5 μmoles of ATP, 138 μmoles of Tris—HCl (pH 7.6), 100 μmoles of mercaptoethanol in a final volume of 1 ml.

The specific exchange rates were corrected for background exchange (ranging from 0.9 to 2.2 μmoles PP/hour/mg DNA) and indicated on the ordinate of the diagram.

Each point represents the result of an incubation with one of the following amino acids: leucine (○), lysine (●) or valine (×)

not to be appreciably altered in the first 48 hours of testosterone action or in the first six days following castration (Tóth, 1968a).

As seen in Fig. 5a, following castration the activities of the leucine, isoleucine, methionine and valine activating enzymes decreased exponentially with a half time of about 1.5 days. When testosterone was injected to rats castrated a fortnight before (Fig. 5b) a lag period of about 12 hours was observed to precede the

progressive increase in activities of the above enzymes. Differences in the rates of increases were also observed, especially in the case of the leucine and valine activating enzymes. Enzymes prepared from the seminal vesicles of castrated rats pretreated with testosterone for 48 hours, stimulated the leucine dependent ATP–PP exchange reaction nearly four times as rapidly as enzymes from the glands of untreated castrates. On the other hand, the exchange rate with valine as substrate was only doubled.

It has been reported previously from this laboratory (Mányai, 1964a) that a considerable part of the proteins of the vesicular secretion of rats is formed by a strongly basic protein. This protein was shown to be rich in lysine (85 μmoles/100 mg protein) and to contain about 40 μmoles/100 mg protein of leucine and valine, respectively (Mányai et al., 1965). Similar proportions of the amounts of these amino acids were also found in the neutral protein which is the other major component of the secretory proteins. In the course of our studies we wondered whether a parallelism could be found between the amounts of these particular amino acids in the secretory proteins and the augmentation of their activating enzymes in the vesicular tissue of testosterone treated castrates. Therefore, the responses of the leucine, valine and lysine activating enzymes to testosterone were studied in castrated rats. As seen in Fig. 6 the rate and extent of the initial increase of activities of these enzymes did not reflect such kind of parallelism. On the contrary, the rate of increase in activity of the leucine activating enzyme exceeded slightly that of the lysine activating enzyme.

Does testosterone regulate the levels of amino acid activating enzymes in the vesicular tissues?

Thus far reference has been made only to the activity of amino acid activating enzymes. This implies that there may be a way to influence the "effective concentration" of these enzymes other than an increase in the levels of enzyme molecules in the tissue due to testosterone treatment, or a decrease of their levels in the absence of this hormone. Obviously the most reasonable explanation for the alteration of enzyme activities investigated here is that the rate of synthesis of the enzyme molecules has been changed as a result of castration or androgen treatment, and this change in the synthetic rate led ultimately to altered levels of the enzyme molecules. The results of the experiments (Fig. 7) are in agreement with this view.

Castrated rats were injected with a single dose of testosterone and the rate of in vitro incorporation of ^{14}C-valine into proteins and the amount of the 105 000 g supernatant protein were determined at various intervals after the injection. The pattern of increase in the "soluble protein" content of seminal vesicles as a function of the time of hormone effect was just the same as in the case of the individual amino acid activating enzymes. Furthermore, the beginning of accumulation of the supernatant proteins as well as the first measurable rise in enzyme activities took place together with a rapid increase in the rate of protein synthesis.

Here should be mentioned our finding that Actinomycin D given to the animals 6 hours subsequent to the testosterone injection abolished the rise in enzyme activities nearly completely (Tóth, 1968).

In further experiments the possibility of activation or inhibition of the amino acid activating enzymes by low molecular weight substances in the course of the

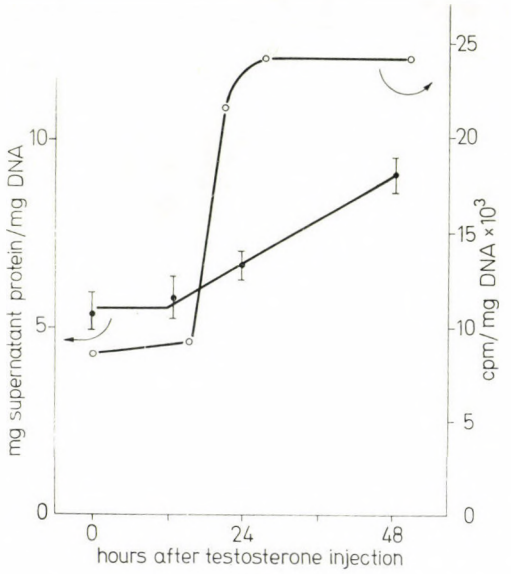

Fig. 7. The accumulation of "soluble proteins" and the acceleration of protein synthesis in the seminal vesicles of castrated rats following testosterone injection

Groups of castrated rats (3 per group) were injected with testosterone at different times before killing, as indicated on the abscissa. Minces of the seminal vesicles were incubated with ^{14}C valine as described previously (Mányai, 1964) for 40 min. The mince was homogenized then with ice cold 0.4 N perchloric acid. The acid precipitates were washed successively with cold perchloric acid, ethanol, a mixture of ethanol-ether (3 : 1) at 60 °C, ether and the total radioactivity of the resulting dry powder (amounts of the nucleic acids were taken into correction) was determined. Incorporated activity was referred to 1 mg of DNA and represented as open circles in the diagram.

Solid circles: protein content of vesicular 105 000 *g* supernates. Each point represents the statistical evaluation of six determinations. The standard deviations from the mean values are shown by brackets

testosterone action was considered. Although the rate of the amino acid dependent ATP–PP exchange has not yet been found to be influenced by addition of the hormone in vitro, several organic compounds were reported to be effective activators or inhibitors of this enzyme reaction (McCorquodale, Mueller, 1958; Hele, 1961, 1964, cf. Novelli, 1967). The relatively slow, progressive rise in the activities of aminoacyl activating enzymes following testosterone administration

is apparently not characteristic of an enzyme activation process. Moreover, similar patterns were found even when different concentrations of Mg^{2+} were used (Figs 3 and 5) or when mercaptoethanol (an activator) was also included in the incubation media (Fig. 6).

Conclusive evidence was obtained in this respect when the additivity of the stimulatory effects of vesicular supernates from castrates and testosterone treated castrates, respectively, was studied on the rate of the ATP–PP exchange reaction (Table 1). Within reasonable limits good additivities were found, suggesting no significance of activators or inhibitors in the testosterone effect observed on the activities of various amino acid activating enzymes.

Table 1

The additive stimulation of ATP—PP exchange reaction by the mixed vesicular supernates of castrated rats and of testosterone treated castrates

Ten animals were sacrificed two weeks after castration. Four of them were injected with Retandrol 48 and 24 hours before killing. The seminal vesicles were homogenized with a mixture of Tris—HCl (0.02 M) and mercaptoethanol (0.001 M) and the 105 000 *g* supernates were prepared. Supernates were diluted with buffered mercaptoethanol (0.25 M). Incubation mixtures contained: ^{32}PP (3 μmoles, 328 000 cpm), $MgCl_2$ (8 μmoles), mercaptoethanol (100 μmoles), protein 174 μg (A) or 184 μg (B), a mixture of L-amino acids (1 μmole of each) or 4 μmoles of leucine, 6 μmoles of valine, respectively, and other components as described in Methods, in a volume of 1 ml. Background exchange: 212 (A), 420 (B), 355 (C) cpm/μmole ATP. Radioactivity was measured utilizing the Cerenkov radiation, in a Liquid Scintillation Spectrometer.

Enzyme preparation	c. p. m./μmole ATP measured with		
	a mixture of 20 amino acids	leucine	valine
A. Castrates, 0.4 ml	1643	403	332
	1568	416	326
B. Castrates injected 48 hours earlier with testosterone, 0.4 ml	3245	985	393
	3245	1020	455
C. Mixtures of 0.2 ml A +0.2 ml B	2510	655	360
	2440	783	404
Mean value of A + B	2425	706	377

Discussion

The regulation of the activity of individual amino acid activating enzymes in the rat accessory sexual glands by androgens has not yet been studied in detail. Experiments with various androgen dependent organs of the guinea pig have revealed that following castration the activities of the amino acid activating enzymes of the seminal vesicles is decreased most markedly (Kochakian et al., 1961).

It has also been reported in the same paper that under the effect of testosterone treatment of castrates the specific enzyme activities are gradually restored to the normal levels. The rate of ATP—PP exchange stimulated by the vesicular supernate has been measured in these experiments mostly in the presence of various mixtures of amino acids, therefore only the sum of the activities of different enzymes has been obtained.

The material presented in this paper made it possible to analyze the effect of deprivation or administration of testosterone on different amino acid activating enzymes of the seminal vesicle of the rat. Our results suggest that testosterone regulates the level of these enzymes in the gland tissues and activation or inhibition of the enzyme activities may not play a significant role in the regulation. In more recent experiments in this laboratory, testosterone was found to accelerate the synthesis of various activating enzymes without influencing their degradation (Tóth, 1968).

After castration the activities of leucine, isoleucine, methionine and valine activating enzymes decrease much more rapidly than the "soluble protein" content of the vesicles. The extent of the decrease is about the same in the case of the four enzyme activities tested and the disappearance of these activities is exponential with a half time of about 1.5 days. This finding suggests a first order degradation reaction for these enzymes in the seminal vesicles of castrated animals.

Following testosterone injection to castrated rats an increase in the activity of the amino acid activating enzymes can be observed, preceded by a lag period of about 12 hours. At this point the simultaneous and progressive accumulation of different amino acid activating enzymes begins but the rate of accumulation of the various enzymes is quite different. Among the enzymes tested the responses of the leucine and valine activating enzymes to testosterone are the extremes in this respect.

The increase in the leucine activating enzyme is about four times as rapid as the accumulation of the total "soluble protein", while that of the valine activating enzyme is nearly the same. This difference is reproducible throughout various incubation conditions and whether the 105 000 *g* supernates or the total homogenates of the vesicles (Tóth, 1968) are used as enzyme preparations. Furthermore, a very similar situation can be found when the change in the concentrations of these enzymes in the vesicular supernate is followed along the time axis of development of the seminal vesicle. The onset of the growth of this organ initiated by endogenous testosterone is accompanied by a different augmentation of leucine, isoleucine, methionine and valine activating enzymes, respectively.

Experiments with rat uterus indicate that as an early effect of estrogen injection to ovariectomized rats a differential increase in at least seven amino acid activating enzymes occurs (McCorquodale, Mueller, 1958; Mueller et al., 1958). It appears that the increased level of the activating enzymes observed in the uterus or in the seminal vesicles is not related to the primary action of the hormones (Williams-Ashman, 1965a; Ching-Sung-Teng, Hamilton, 1967). However,

their optimal concentration seems essential for the growth and function of these organs. From this point of view it is of interest that although no increase in activating enzymes of rat liver following corticosteroid injection was found (Smith et al., 1961), in regenerating liver these enzymes were increasing (Hultin, von der Decken, 1958). The growth of various organs involves some increase in these activating enzymes, however, this latter process seems to be more intensive in the seminal vesicles than in other androgen or estrogen dependent organs or in the regenerating liver.

Obviously, the differential induction of various amino acid activating enzymes by testosterone may serve as a basis for some attractive hypotheses. However, the problem of the differential inactivation of certain activating enzymes or the possibility of their differential binding to the cellular particles seriously interferes with any interpretation of the results presented in this paper. It should be kept in mind that the response of activating enzymes to testosterone in different cell types of the vesicles may vary. In addition, the presence of enzyme-bound activators or inhibitors, influencing the rate of ATP–PP exchange reaction, cannot be excluded.

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The Muscle Protein Fibrillin

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1. A method for the isolation of the muscle protein fibrillin has been described. After the successive removal of the water soluble proteins, myosin, tropomyosin and actin, a solution containing 1.0 M KI, 10 mM $Na_2S_2O_3$, 10 mM Na_2HPO_4, 10 mM ATP (pH 7.5) has been used for fibrillin extraction.

2. Some physical and chemical properties of the purified protein have been described: e.g. solubility, amino acid composition, etc. . . .

Fibrillin dissolved in a solution containing 1.0 M KCl (pH 7.5) has a $s^0_{20,w} = 4.60$ S, $D^0_{20,w} = 4.36 \times 10^{-7}$ cm^2/sec. The molecular weight calculated by Svedberg's equation is 110 000 $\pm$ 10 per cent.

3. It is suggested that fibrillin plays a structural role in the ultrastructure of myofibrils.

Introduction

In recent years the role of structural proteins in the ultrastructure of the striated muscle has been investigated in detail (Harsányi, Guba, 1963; Guba et al., 1964; Ebashi, 1968).

All the hypotheses on muscle contraction agree that one of the two filamentary systems contains myosin (primary filaments) and the other contains actin (secondary filaments) (Huxley, Hanson, 1960; Davies, 1963).

The investigations on isolated primary and secondary filaments seem to support this view (Hanson, Lowy, 1963; Huxley, 1963).

On the other hand, there are some observations inconsistent with this view, e.g the discrepancy in the quantity of the extractable amount of myosin and actin related to the estimated amount of these proteins in the myofilamentary system (by electron microscopy). In previous experiments we have tried to give an account on the whole protein content in the myofibril and on the individual proteins in respect to their location (Guba et al., 1966).

It has already been shown that there exists a new structural protein, the fibrillin which seems to give the basic filamentous structure of the myofibrils (Guba et al., 1964; Guba, 1968). Fibrillin was first isolated with an alkaline urea-containing solution and its physico-chemical properties were investigated (Guba,

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Harsányi, 1964; Guba et al., 1964). Since urea even in a low concentration affects the conformation of proteins, another extraction method was needed.

A detailed study of this extraction method and the physico-chemical characterization of the purified protein is presented.

Materials and Methods

In the experiments minced muscle of rabbit was used. Care was taken to keep the muscle in the relaxed state. The extractions were carried out between 0–5 °C using gentle mechanical stirring. In some cases a Waring Blendor was also used.

Solutions

Soln.* 1. 0.3 M KCl, 0.15 M phosphate buffer, 5 mM ATP (pH 6.5)
Soln. 2. 0.47 M KCl, 10 mM $Na_4P_2O_7$, 10 mM phosphate buffer (pH 6.1–6.3) (Hasselbach–Schnieder soln.)
Soln. 3. 0.2 mM ATP, 0.2 mM ascorbic acid (pH 7.0–7.4)
Soln. 4. 0.2 mM ATP, 0.2 mM ascorbic acid, 0.2 mM $NaHCO_3$, 2.0 mM Na_2CO_3 (pH 8.0)
Soln. 5. 0.1 M KCl, 1 mM $MgCl_2$ (pH 7.0)
Soln. 6. 1.0 M KI, 10 mM $Na_2S_2O_3$, 10 mM Na_2HPO_4, 10 mM ATP (pH 7.5)
Soln. 7. 50 mM $NaHCO_3$
Soln. 8. 0.1 M KCl (pH 4.5)
Soln. 9. 1.0 M KCl, 10 mM Tris buffer (pH 7.5)
Soln. 10. 50 mM KCl, 0.2 mM cysteine (pH 7.0)
Soln. 11. 2 mM $MgCl_2$, 10 mM Tris buffer, 5 mM ATP (pH 7.0)
Soln. 12. 0.5 M KCl, 2 mM $CaCl_2$, 10 mM Tris buffer, 5 mM ATP (pH 7.0)

All the chemical reagents used in the experiments were of A.R. grade, products of the Firm Reanal (Hungary).

Protein content

Protein concentrations were determined by the biuret method of Gornall et al. (1949), calibrated with the Kjeldahl technique, based on a nitrogen content of 16 per cent.

Measurement of ATPase activity

The enzyme activity of actomyosin was determined in soln. 11. That of myosin was determined in soln. 12. The temperature of incubation was 20 °C.

* Abbreviations used: I, ionic strength; mol., molecular; ppt., precipitate; soln., solution; vs., versus; vol., volume; wt., weight.

The amount of the split P_i was measured by the colorimetric method of Fiske and Subbarow (1925). The activity was calculated for μM P/min/mg prot. ATP sensitivity was calculated from viscosity data (Portzehl et al., 1950):

$$\frac{\log \eta_{rel} - \log \eta_{relATP}}{\log \eta_{relATP}} \cdot 100$$

Determination of myosin content

The extracts were dialysed twice for 24 hours each time against 20 volumes of soln. 10, changed three times. The precipitated myosin was used for the measurements.

Estimation of actin content

After the extraction of myosin an acetone powder was made from the residue. The powder was extracted three times with 20 volumes of soln. 3 for 10 minutes at low temperature. The salt concentration was adjusted to 0.1 M KCl and 1 mM $MgCl_2$ (pH 7). After standing at room temperature for 30 minutes the gel was spun down at 100 000 *g* for 2 hours. The F-actin content of the sediment was measured.

Detection of tropomyosin

After an $(NH_4)_2SO_4$ fractionation the protein was crystallized according to the method of Bailey (1948). The crystals were observed in a light microscope or in the electron microscope. Sometimes the protein was subjected to ultracentrifugation for further characterization.

Myokinase test for water soluble proteins

The usual superprecipitation test was made as for actomyosin but instead of ATP, ADP was used. A positive test for superprecipitation indicates the presence of myokinase (Guba, 1950).

The amount of water soluble proteins in the different extracts was measured after the precipitation of the structural proteins.

Results

Preparation of the protein

a) *Extraction of myosin.* Fibrillin was extracted after the successive removal of the other structural proteins. First step was the removal of myosin.

To this end the fresh minced muscle was extracted for 30 minutes with 5 volumes of soln. 1. The suspension was spun down (3 000 rev/min for 10 minutes).

The protein content, myosin content and the ATPase activity of the supernatant and that of the residue were measured.

According to our investigations – after an inserted Waring blending – four extractions mentioned above were enough to remove all the myosin. During this procedure tropomyosin dissolved too, but incompletely. The extraction of the water soluble proteins was checked by the myokinase test.

After a complete myosin extraction, from one part of the residue an acetone powder was made to determine its actin content. The extracted amount of actin during the successive treatment with soln. 1 seemed to be negligible.

b) *Removal of actin and tropomyosin.* The residue of muscle after four extractions with soln. 1 was washed with 10 volumes of soln. 7 for 30 minutes. After it was spun down, the sediment was washed two times with 10 volumes of deionized water containing 10 μM ATP (pH 7). The ATPase activity of the residue was checked. In consequence of washing the rate of hydration of the muscle diminished.

To the residue 5 volumes of soln. 4 were given (to depolymerize actin) and after one minute of homogenizing in the Waring Blendor it was slowly stirred overnight. Next day this step was repeated, and the residue was spun down for 20 minutes at 10 000 g. The supernatant fluids were checked for actin, tropomyosin and fibrillin. It was found that during these two steps all actin and tropomyosin were extracted.

c) *Extraction of fibrillin.* The residue after the removal of myosin, actin and tropomyosin was washed with 5 volumes of soln. 8.

After this the sediment was extracted with 3 volumes of soln. 6 overnight. Next day this step was repeated. The two collected extracts were dialysed against 100 volumes of soln. 8. During dialysis 75 per cent of the extracted proteins precipitated.

By slow precipitation fibrillin formed well ordered aggregates which seem to be crystalline-like in the light microscope.

The precipitate was spun down at 26 000 g. The collected protein was redissolved by dialysis against 2 volumes of soln. 9. This purification procedure was repeated two more times. The amount of the protein after purification was about 70 per cent of the KI extracted material. A summary of the preparation procedure is shown in Table 1.

Characterization of the protein

For the physico-chemical characterization of the protein a solution of a three times precipitated fibrillin was used.

a) *Chemical data.* The chemical data of the KI extracted and of the urea extracted protein are identical (N-content: 15.5 per cent, lipid content about 5 per cent).

b) *Amino acid composition.* The amino acid composition of the protein after hydrolysis with 6 N HCl was determined in a Beckman automatic amino

Table 1

Schedule of preparation (7 expts.)

Starting material: 300 g fresh, minced muscle, total protein content: 61.5 ± 5 g, total splitted P_i 14.5 mM P_i/min

	Extraction	Dissolved substance				Residue					
		Protein extracted		P_i split		ATP sens.	Tropo-myosin	Wet weight	Protein content	Splitted P	Actin* extr.
		Totals	Precipitate	Totals	Precipitate						
Extraction of myosin	I. Soln. 1.	50% (30.7±1.0 g)	31.0% 19.4±1.0 g)	76% (11.0 mM P_i/min)	70% (10.2 mM P_i/min)	0	—		50% (31.0± ±1.6 g)	25% (3.6 mM P_i/min)	100% 5.8 g
	II. Soln. 1.	8.5%	5.5%	14%		5	+		42%	10%	100%
	III. Soln. 1.	3.0%	1.5%	10%		10	+		38%	3%	97%
	IV. Soln. 1.	2.0%	0.5%	3%		30	+	200 g	35% (21.6±	— ±1.5 g)	95%

		Dissolved substance (extracted amount of proteins)				Residue		
		Totals	Actin	Tropomyosin	Fibrillin	Wet weight	Protein content	Actin* extr.
Extraction of tropomyosin and actin	V. Soln. 7.	1.0%	—	—	—		34%	—
	VI. Soln. 4.	12.0%	9.5%	+	—		22%	40%
	VII. Soln. 4.	8.0%	7.5%	+	—	56 g	14% (8.7±0.7 g)	10%
Extraction of fibrillin	VIII. Soln. 8.	1.0%	—	—			13%	
	IX. Soln. 6.	6.0%	?	—	4.5%		8%	
	X. Soln. 6.	3.0%	—	—	3.0%		6% (3.5±0.5 g)	

* The extractable amount of actin was related to the residue of the first extraction.

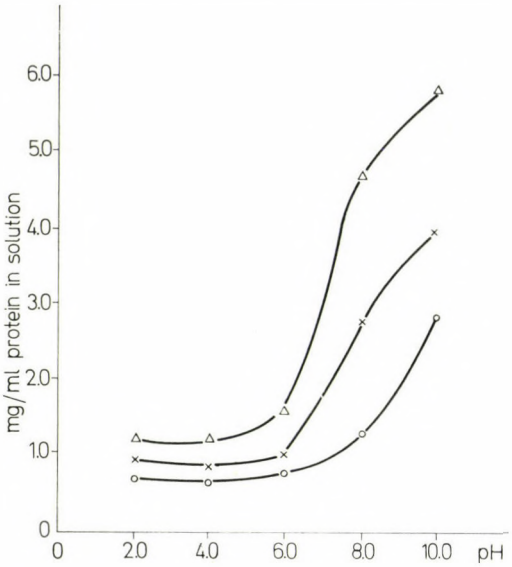

Fig. 1. The solubility of fibrillin as a function of pH and salt concentration, Buffer $I = 0.05$
△ — △ — △ 1.0 M KCl + buffer
× — × — × buffer
○ — ○ — ○ 0.1 M KCl + buffer

acid analyzer. The data have been calculated for g mole $/10^5$ g protein. Cys/2 1, Asp 81, Thr 61, Ser 53, Glu 86, Pro 57, Gly 82, Ala 67, Val 50, Met 38, Ile 54, Leu 60, Tyr 34, Phe 40, His 19, Lys 51, Arg 54, Try 10, Sarcosine 4, Total: 902. Polar groups: 440. To prove that sarcosine is not a product of decomposition further experiments are needed. A comparison of the proportion of polar to apolar amino acids in fibrillin related to other structural proteins clearly shows its apolar character.

c) *Solubility*. The solubility of fibrillin is a function of the pH and ionic strength as shown in Fig. 1. Fig. 1 shows that the protein is soluble at an alkaline pH and a high ionic strength. Even under these conditions the solubility is 6 mg/ml. The protein is least soluble at an I value of about 0.1 (pH 4.5). The solubility curve is demonstrated in Fig. 2. It suggests that the protein is homogeneous. As the highest solubility is at an I value of about 1, at a moderate alkaline pH, the molecular parameters of the protein were determined in soln. 9.

d) *Electrophoretic behaviour*. Electrophoresis was performed on a cellulose acetate strip of 25×122 mm. Conditions: veronale acetate buffer according to Michaelis, $I = 0.15$; 10 V/cm, 15 mA.

Azocarmin staining was used. Most of the protein remained at the start point and a single component appeared (Fig. 3).

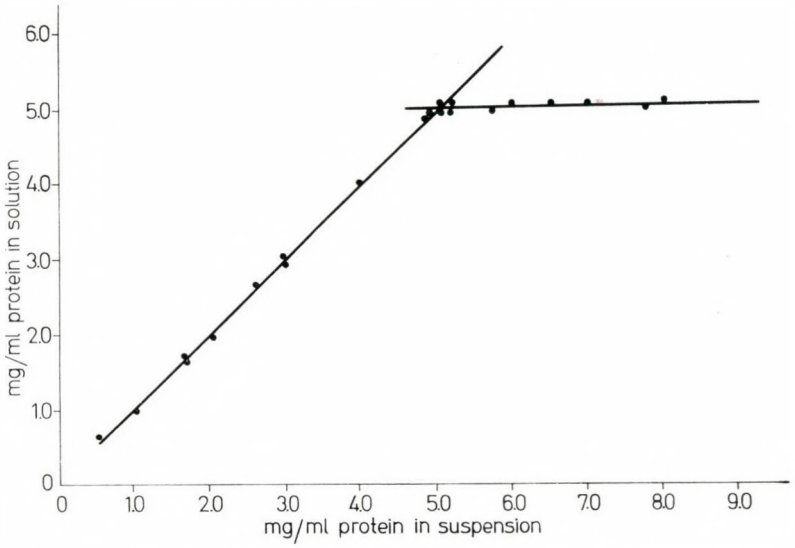

Fig. 2. Solubility curve of fibrillin in soln. 9

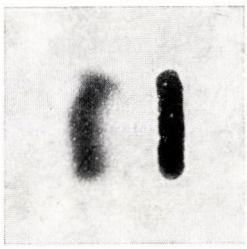

Fig. 3. Gel electrophoresis on cellulose acetate strip at pH 8.6 (Michaelis buffer)

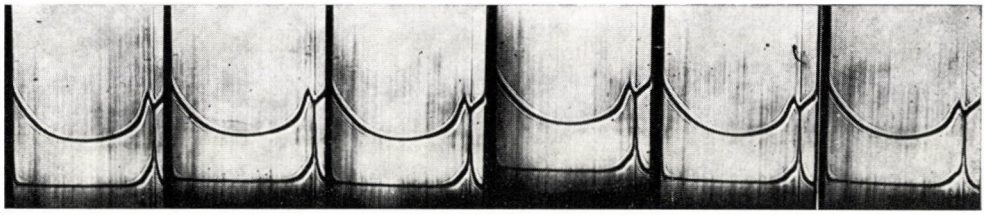

Fig. 4. Sedimentation pattern of fibrillin: 18 min. after reaching 50 740 rev/min. Bar angle 70°.
Upper curve in soln. 6 c = 3.5 mg/ml
Lower curve in soln. 9 c = 4.7 mg/ml

e) *Sedimentation experiments.* In the ultracentrifuge the protein gave a single peak (Fig. 4). In the upper curve the solution contained 1.0 M KI and therefore the base line is concave.

Fig. 5 shows the sedimentation coefficient vs. concentration.

$$s^0_{20,w} = 4.60\ (\pm\ 0.08)\ \text{S}$$

f) *Diffusion experiments.* The diffusion coefficient vs. concentration is presented in Fig. 6.

$$D^0_{20,w} = 4.36\ (\pm\ 0.08)\ 10^{-7}\ \text{cm}^2/\text{sec}$$

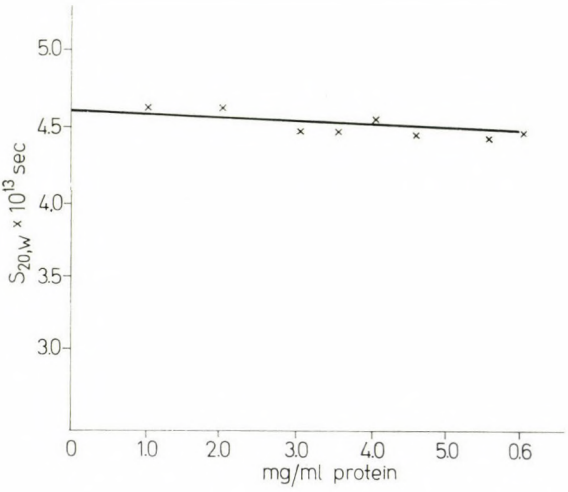

Fig. 5. $s_{20,w}$ plotted vs. protein concentration

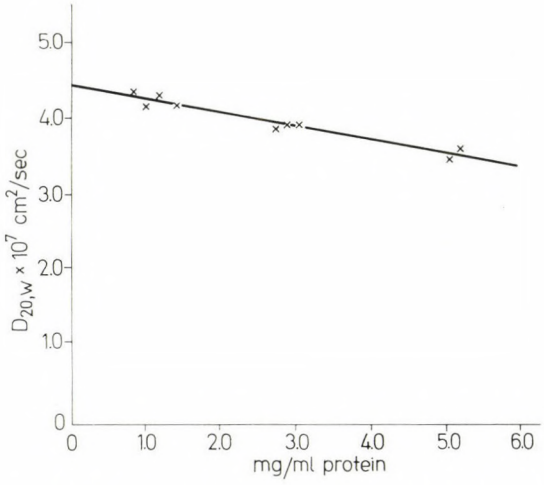

Fig. 6. $D_{20,w}$ plotted vs. protein concentration

g) *Viscosity measurements.* In Fig. 7 the specific viscosity vs. protein concentration is shown. The intrinsic viscosity:

$$\lim_{c \to 0} \frac{\eta_{spec}}{c} = 0.25 \text{ dl/g}$$

h) *Electron microscopy.* For electron microscopy the negative staining technique was used. As mentioned above, during slow precipitation fibrillin forms well ordered aggregates. The aggregates are crystalline in nature as revealed under the light microscope (Fig. 8) and seem to be ordered aggregates in the electron

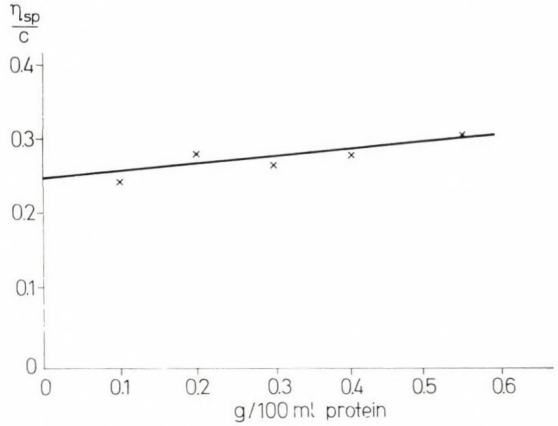

Fig. 7. Specific viscosity plotted vs. protein concentration

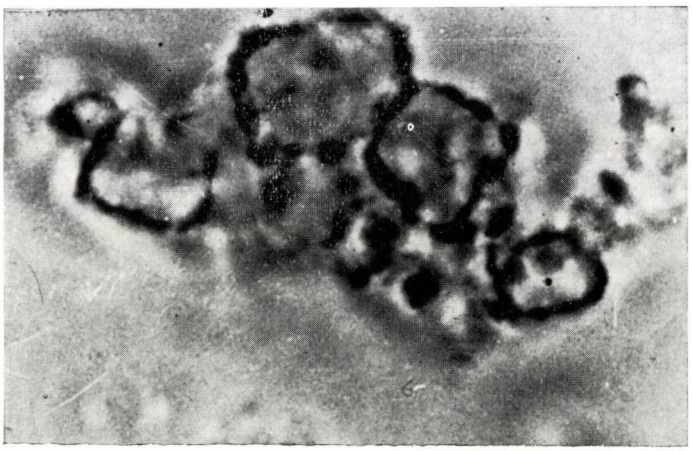

Fig. 8. Aggregates of fibrillin obtained by slow precipitation of the protein. Light microscope photograph. 600×

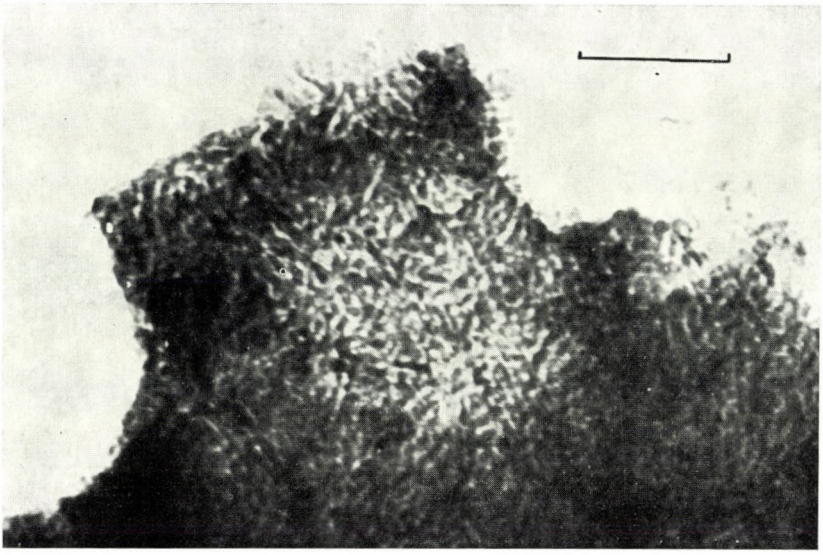

Fig. 9. Micrograph of a fibrillin aggregate observed in the electron microscope. Negative staining. 21 000×

microscope, too. Nevertheless, the aggregates are not real three dimensional crystals. The formation shows a well ordered filamentous structure (Fig. 9). The filaments are twisted and they close an angle of about 30°.

i) *The shape and size of the molecule.* The value of the sedimentation and diffusion constants, the viscosity data and the electron micrographs as well allow some calculations on the molecular weight and the size and shape of the fibrillin molecule.

The molecular weight was found to be 110 000 ± 10 per cent based on Svedberg's equation ($V = 0.731$ ml/g; $\varrho = 1.053$ g/ml). The shape is an elongated ellipsoid with an axial ratio of 1 : 20. The diameter of the molecule is about 20 Å.

Discussion

The extreme solubility of fibrillin makes its characterization difficult. Most likely, this is the reason why it has been isolated only recently, despite the fact that it occurs in relatively high amounts in myofibrils.

The amino acid composition of fibrillin is somewhat similar to that of actin. Among the known structural proteins fibrillin has the lowest content in polar amino acids. This property seems to be connected with its low degree of solubility.

The amounts of polar groups in structural proteins are as follows: tropomyosin > myosin > actin > fibrillin.

The relatively high Pro content suggests that in the peptide chain of fibrillin there exists a low α-helix content.

The fibrillin molecule is of an elongated shape, and has a defined affinity for screwing on. The relation of fibrillin to other and recently discovered structural elements, e.g. natural tropomyosin, α and β actinin etc. (Ebashi et al., 1964; Ebashi, Ebashi, 1964; Maruyama, 1965; 1965a) has not been investigated so far.

There is a lack of evidence for any enzyme activity of fibrillin up to the present.

No experimental evidence has been obtained so far for any interaction between fibrillin and other proteins of the myofibril.

Our observations (Guba et al., 1964), that after the removal of myosin, tropomyosin and actin there still exists a well defined filamentous structure disappearing after the extraction of fibrillin gives a piece of evidence supporting the view that fibrillin is responsible for the basic filamentous structure existing in the myofibril.

Thus its role in the function of the striated muscle is probably a structural one.

It should be mentioned that one of us (F.G.) succeeded in isolating a fibrillin-like protein from the sperm tail (Guba, 1966).

It is attractive to suppose that some fibrillin-like protein is common in all contractile biological systems and lends them the basic filamentary structure.

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Thermal Decomposition of Glycosaminoglycans

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Glycosaminoglycans of different origin and some of their monosaccharide units were investigated derivatographically and by means of a visual DTA instrument.

According to the thermal decomposition curves it could be concluded that the thermal properties of glycosaminoglycans are very similar: in all the investigated compounds a characteristic decomposition process took place at about 240 °C that was accompanied by a weight loss of the same order. The process was not affected when the polysaccharide was bound to protein. The decomposition of the polysaccharide molecules took place always in solid phase, while that of their monosaccharide units occurred in molten phase.

Thermoanalytical methods are widely used for the structural investigation of inorganic compounds, but very few studies are available on the thermal properties of naturally occurring organic materials. This may be partly due to the difficulties caused by a variation of samples of biological origin and to the fact, that identification of thermal processes is hindered by the coexistence of organic and inorganic compounds in the experimental system. In spite of this, the method of differential thermal analysis has successfully been used in investigations of enthalpy changes accompanying the denaturalization of proteins (Steim, 1965) and the thermal decomposition of polysaccharides (Chesters, Thompson, 1961).

The object of our present experiments was to study the thermal behaviour of glycosaminoglycans of different origins, and of some of their monosaccharide units and to find an adequate method for their identification in natural complexes. These investigations were motivated by the recently increasing interest in the glycosaminoglycans of connective tissue. In addition some other polyanions of the same structural type were studied.

The investigations were carried out by means of a complex thermoanalytical method, derivatography, developed by Paulik et al. (1958). The derivatograph measured and recorded simultaneously the weight change (TG curve), rate of weight change (DTG curve), enthalpy change (DTA curve) and the temperature (T) of the same sample.

Materials and Methods

Glycosaminoglycans and carbohydrates

The glycosaminoglycans were extracted from human, dog, rabbit and pig aortic intimas by a modification of the method of Dyrbye and Kirk (1957), as described previously (Dévényi et al., 1963). A parallel batch of human aortic glycosaminoglycans was prepared by the method of Antonopoulos et al. (1965). The hexosamine content was determined by Rimington's modification of the Elson–Morgan method (Rimington, 1940); hexuronic acid by Bitter and Muir's modification of the method of Dische (Bitter, Muir, 1962), and sulfate by Robinson's method (1960). Table 1 summarizes the analytical results obtained with the glycosaminoglycan preparations.

Table 1

Analysis of glycosaminoglycan preparations

	mg/100 mg glycosaminoglycan		
	hexosamine	hexuronic acid	sulfate
human glycosaminoglycan (prepared according to Dyrbye and Kirk)	16.5	8.5	6.0
human glycosaminoglycan (prepared according to Antonopoulos)	18.5	11.0	7.5
dog glycosaminoglycan	15.0	8.5	5.5
rabbit glycosaminoglycan	15.0	9.0	6.0
pig glycosaminoglycan	13.0	8.0	3.5

The following individual glycosaminoglycans and other polyanions were used in the experiments: chondroitin sulfate (Light, Colnbrook). hyaluronic acid (Reanal, Budapest), heparin (Richter, Budapest), K-agar (Difco Lab., Detroit), dextran sulfate (Schuchardt, München). A chondroitin-4-sulfate-protein complex was the generous gift of Dr. Helen Muir, London.

D-galactosamine.HCl (Sigma, St. Louis), D-glucosamine HCl (Light, Colnbrook), D-glucuronic acid (Fluka, Buchs), glucuronic acid lactone (Light, Colnbrook) were commercial carbohydrates. D-glucosamine was prepared from D-glucosamine.HCl by passing it through a Dowex-1 (OH^-) column, followed by freeze drying.

Thermal analysis

The studies were carried out using a F. Paulik–J. Paulik–L. Erdey MOM derivatograph. The samples (approximately 100 mg) were weighed into a platinum crucible. The heating rate was 10 °C/min up to 900 °C. To record monosaccharide units a visual DTA instrument (Erdey et al., 1968) was used.

Results and Discussion

Fig. 1 shows the derivatogram of chondroitin-sulfate. The moisture content of the sample, that is released at 80 °C is 18 per cent according to the TG curve. The sample is thermostable up to 200 °C. Decomposition starts at 200 °C and

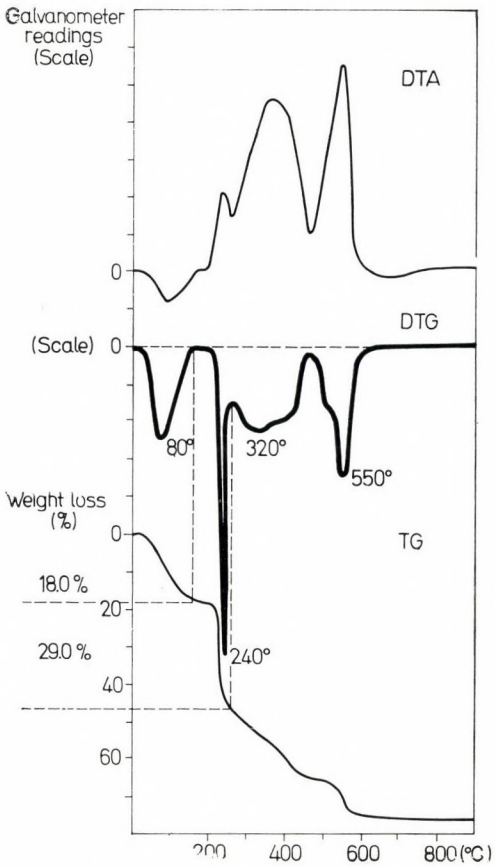

Fig. 1. Chondroitin-sulfate

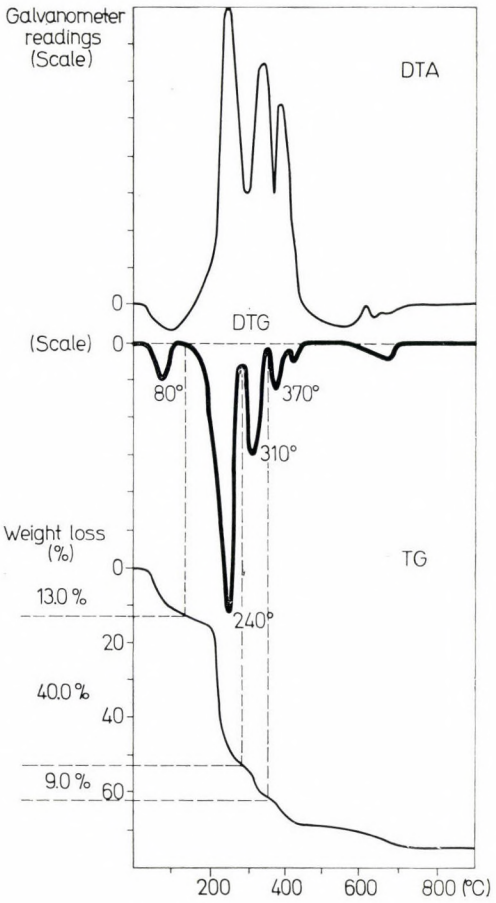

Fig. 2. Hyaluronic acid

reaches its maximum rate at 240 °C. This process is accompanied by a weight loss of about 29 per cent. With increasing temperature decomposition continues at a constant rate and at 320 °C and 550 °C two additional DTG maxima are formed. The previous decomposition is combined with an exothermic enthalpy change; it may be concluded that the enthalpy change is determined by the recombination and burning of the compounds formed during the decomposition process.

On the derivatogram of hyaluronic acid (Fig. 2) after the loss of water content the same sharp DTG maximum is observable as in the case of chondroitin sulfate, proving the similarity of the compounds. On the other hand, the appear-

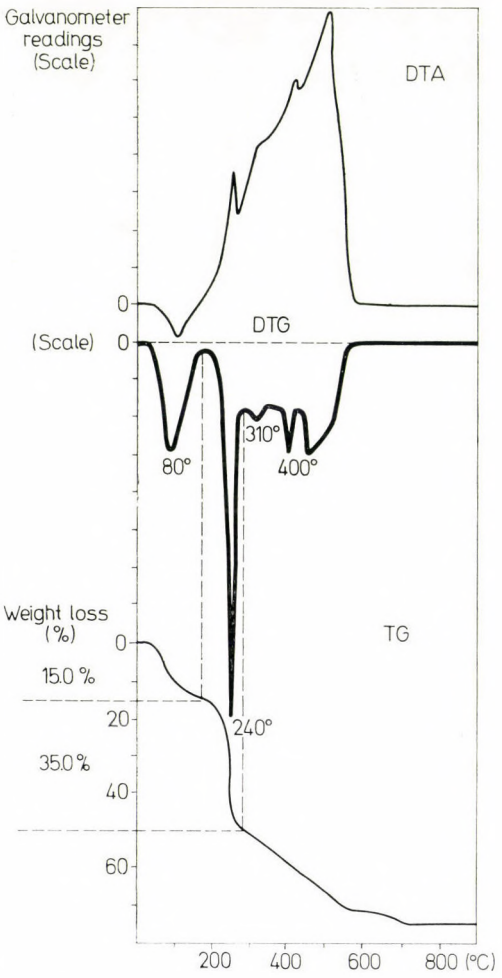

Fig. 3. Mixture of chondroitin sulfate and hyaluronic acid (3 : 1)

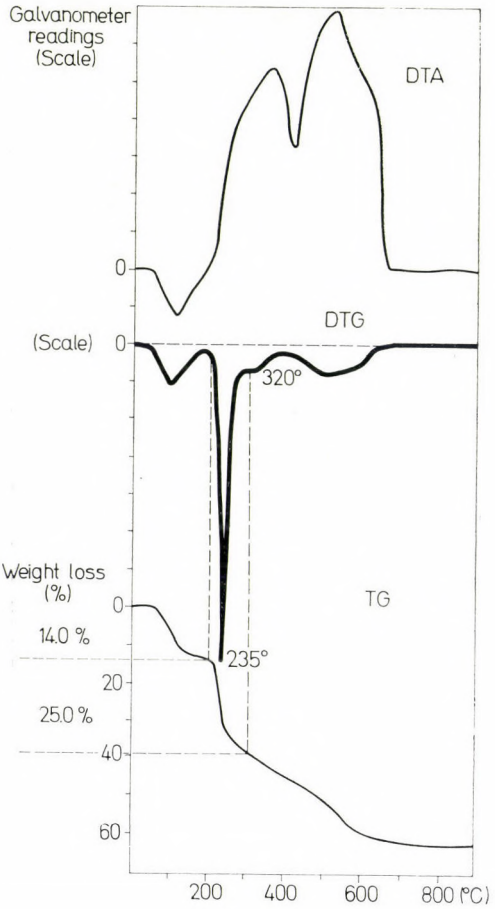

Fig. 4. Heparin

ance of a DTG-peak at 370 °C and the lack of a 550 °C maximum seen in the previous figure, point to structural differences between the two materials.

Fig. 3 shows the derivatogram obtained with a mixture of chondroitin sulfate and hyaluronic acid. It is to be seen that the decomposition process which takes place at 240 °C is dominant.

In Fig. 4 the derivatogram of heparin is presented. The process of decomposition is extremely similar to that of the compounds previously described. The sharp DTG maximum appears at 235 °C.

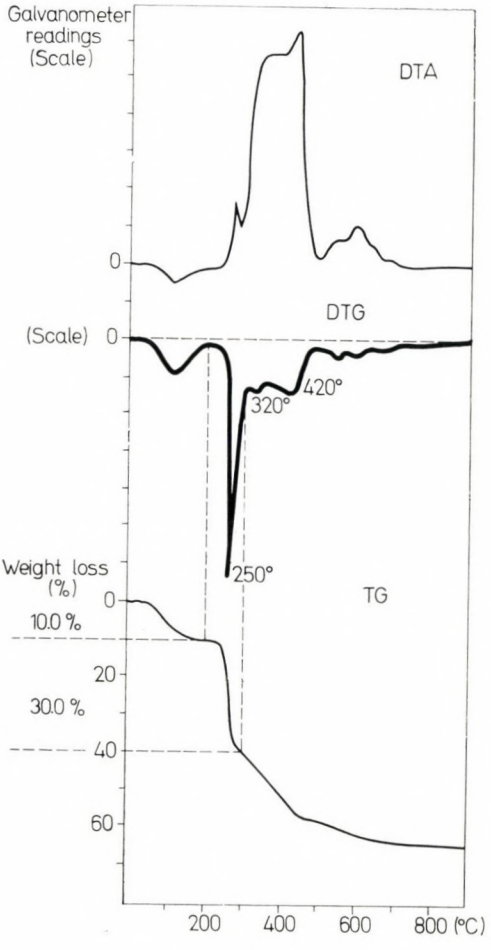

Fig. 5. K-agar

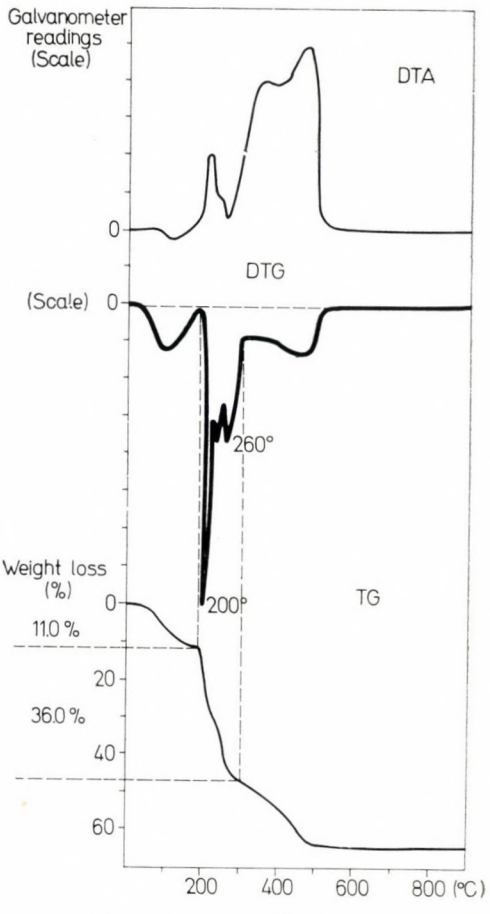

Fig. 6. Dextran-sulfate

In Fig. 5 and Fig. 6 thermal decomposition curves of K-agar and dextran sulfate are to be seen. In the case of K-agar the main decomposition reaction occurs with maximal rate at 250 °C, while in the dextran sulfate sample the process starts already below 200 °C and three peaks are formed in this area.

Fig. 7 shows the derivatograms of two human aortic glycosaminoglycan preparations. On the DTG curve of the glycosaminoglycan mixture prepared according to the method of Dyrbye and Kirk (curves "a") there is a sharp peak

at 220 °C accompanied by approximately 28 per cent weight loss. At 320 °C and 540 °C two additional maxima appear.

The thermoanalytical decomposition curves of aortic glycosaminoglycan prepared by the method of Antonopoulos (curves "b") are in good agreement with those of the other preparation.

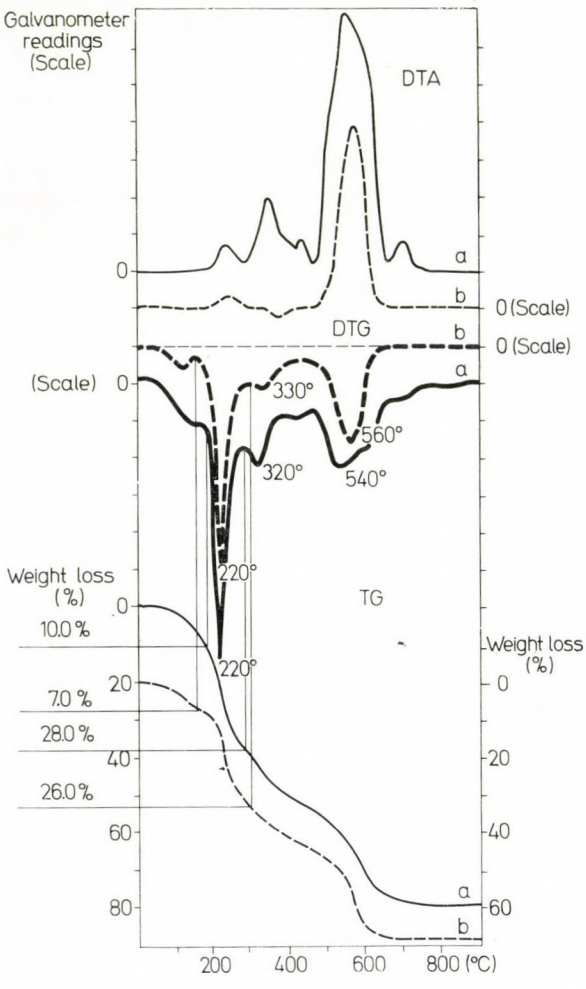

Fig. 7. Human aortic glycosaminoglycans. *a)* prepared according to the method of Dyrbye and Kirk, *b)* prepared after Antonopoulos

In Fig. 8 thermal decomposition of glycosaminoglycans extracted from dog, rabbit and pig aortas is represented. The decomposition of pig and dog aortic glycosaminoglycans (Figs 8/a and 8/b) is similar to that of glycosaminoglycan of

human origin; on the DTG curves obtained with rabbit glycosaminoglycan (Fig. 8/c) two maxima are formed in the discussed temperature area at 210 °C and at 250 °C.

Based on the above reported results it might be concluded that the thermal decomposition of glycosaminoglycans of different origin is very similar and is

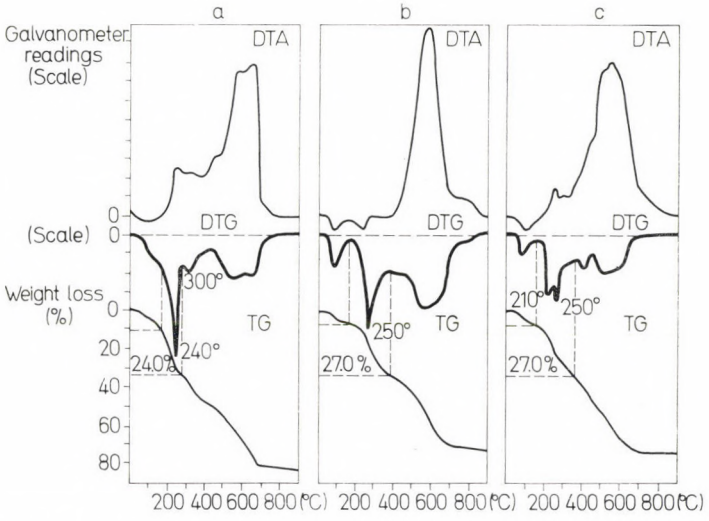

Fig. 8. Aortic glycosaminoglycans of different species: *a)* pig aortic glycosaminoglycan, *b)* dog aortic glycosaminoglycan, *c)* rabbit aortic glycosaminoglycan

characterized by a decomposition peak at about 240° resulting in a weight loss of approximately the same order.

When the polysaccharide molecule is bound to structural protein the decomposition process does not change markedly, as indicated by the derivatogram of chondroitin-4-sulfate-protein in Fig. 9. After the adsorbed water has been released the thermal decomposition of the sample starts at about 180 °C, the rate of weight change being highest at 240 °C. The second decomposition stage is shown by the DTG maximum at 310 °C.

In addition to the above discussed investigations, thermal properties of the monosaccharides building up the carbohydrate chain of the glycosaminoglycan molecules have been studied.

Fig. 10 shows the derivatograms of D-galactosamine.HCl (Fig. 10/a) and D-glucosamine. HCl (Fig. 10/b). The decomposition curves indicate that the process of thermal decomposition of the two carbohydrates is very similar. On the DTA curves at 195 °C an endothermic peak is formed, indicating the melting point and the heat of decomposition. As the decomposition started already at 190 °C, the phase change was followed also by a DTA instrument suitable for visual observa-

tion. Decompositions took place with maximal rate at 200 °C. The rate of the first process of decomposition has a minimum at 280 °C accompanied in both samples by a weight loss of approximately 50 per cent. The derivatograms indicate that,

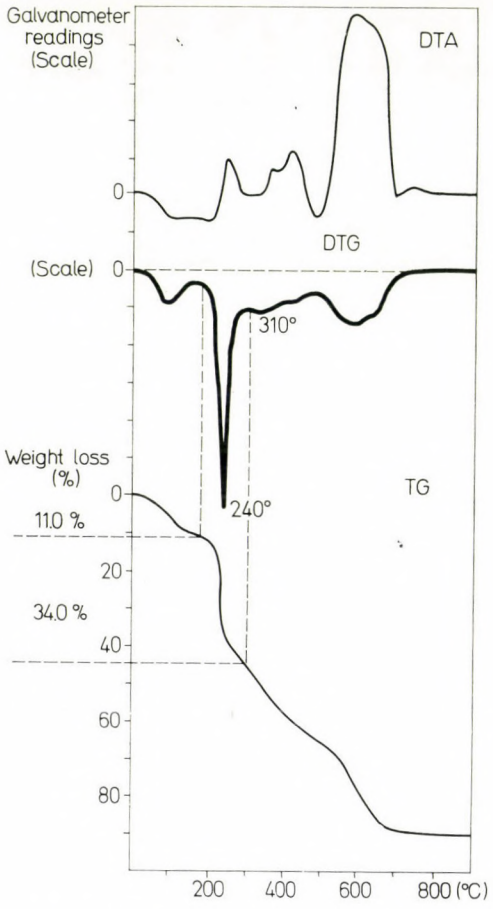

Fig. 9. Chondroitin-4-sulfate protein

in agreement with the thermal decomposition of other monosaccharides (hexoses, pentoses) (Lóránt, Boros, 1965) no temperature interval can be found, where the investigated carbohydrates would keep a constant weight. The carbonization starting above 300 °C is indicated by the flat exothermic DTA curve. This process takes place with an approximately constant rate of weight loss.

In Fig. 11 thermoanalytical decomposition curves of D-glucosamine are presented. The endothermic peak at 115 °C on the DTA curve indicates the melting

of the sample; this is in good agreement with the described melting point of the compound (Bruckner, 1961). Decomposition starts immediately at the phase change and proceeds with maximal rate at 95 °C, 110 °C, 240 °C and 330 °C.

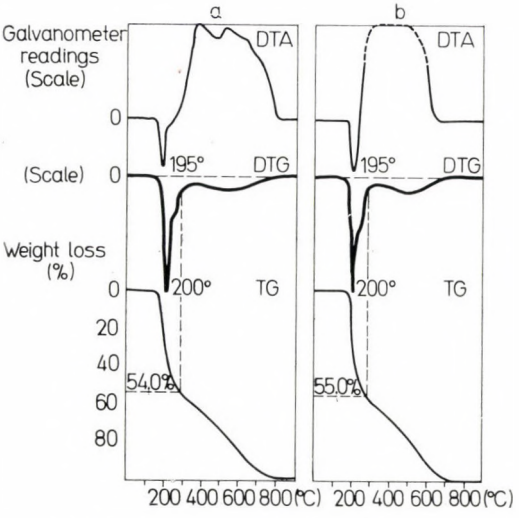

Fig. 10. *a)* D-galactosamine.HCl, *b)* D-glucosamine.HCl

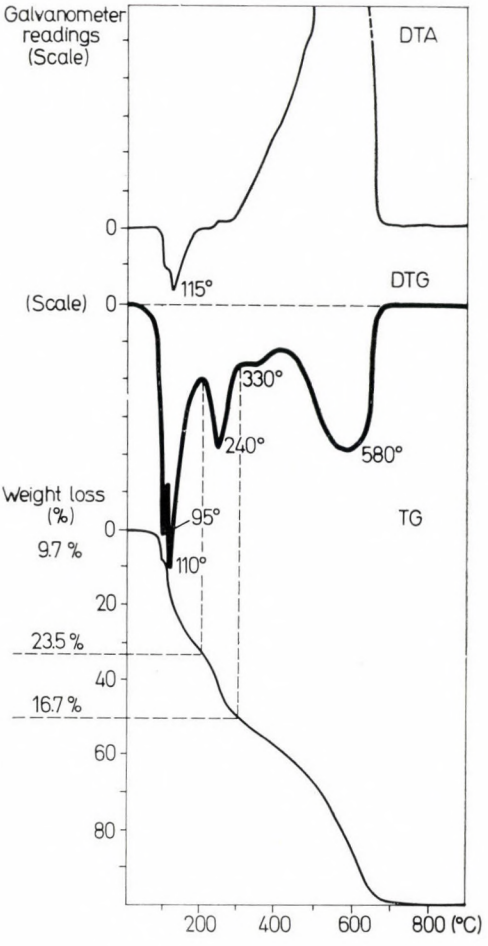

Fig. 11. D-glucosamine

Comparing the derivatogram of D-glucosamine with that of D-glucosamine.HCl (Fig. 10/b) it is to be seen that the release of as little as 1 mole of hydrochloric acid has a great influence on the thermostability of the compound.

Fig. 12 demonstrates the derivatograms of D-glucuronic acid (Fig. 12/a) and of glucuronic acid lactone (Fig. 12/b). The endothermic maxima at 160 °C on the DTA curves indicate the melting and the simultaneous decomposition of the

samples. There is a difference between the shape of the DTA and DTG curves of the two investigated compounds, indicating that the decompositions take place in different ways. These discrepancies, however, cannot be demonstrated quantitatively by the data obtained from the TG curves.

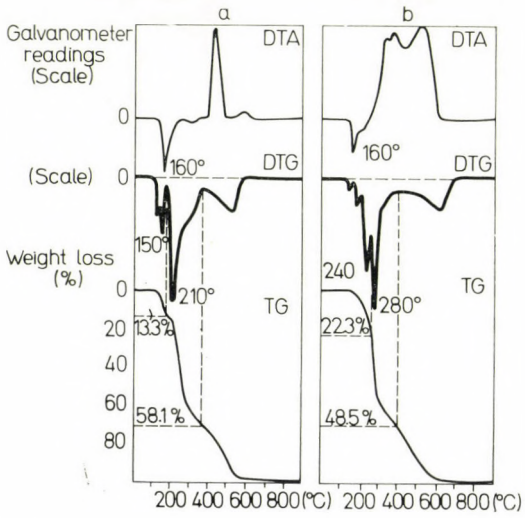

Fig. 12. *a)* D-glucuronic acid, *b)* glucuronic acid lactone

Based on the results reported above it can be concluded that the process of thermal decomposition of the polymer carbohydrates (glycosaminoglycans) is not just the sum of those of their monosaccharide units.

A significant difference was found between the properties of mono- and polysaccharides: the decomposition of the monosaccharide molecules always took place in the molten phase, while in the case of polysaccharides, as a result of the higher thermostability of the polymer chain, the reaction started with depolymerization and it took place in the solid phase.

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Identification of Glycosaminoglycan-β-Lipoprotein Complexes in the Atherosclerotic Aorta Intima by Thermoanalytical Methods

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A thermoanalytical method, suitable for the identification of glycosaminoglycans also in their protein-bound form, was used to find evidence for the *in vivo* existence of glycosaminoglycan-β-lipoprotein complexes in the atherosclerotic intima. The conversion of glycosaminoglycans into an *in vitro* glycosaminoglycan-β-lipoprotein complex resulted in an increase in thermostability and in a significant decrease of a characteristic glycosaminoglycan peak at about 240 °C on the curves indicating the rate of weight change of the sample. When the complex was dissociated by the extraction of the lipid components, the peak became again detectable. The same 240 °C peak could be demonstrated in healthy aorta intimas as a result of the thermal decomposition of structural glycosaminoglycans. The shape of the curves obtained with samples of atherosclerotic intimas (both human and experimental) in the same area resembled that of the curves of *in vitro* glycosaminoglycan-β-lipoprotein complexes. A correlation could be established between the gradual decrease of the peak and the evolution of the atherosclerotic lesions. Derivatograms of delipidated atherosclerotic aorta became similar to those of the healthy tissue.

There is a significant statistical relationship between high serum lipid levels and the clinical manifestation of coronary atherosclerosis. This suggests that some serum lipids may enter the arterial wall and be deposited in the intima, thus leading to the formation of atheromatous plaques. Serum β-lipoprotein has been identified in the arterial wall by immunological methods (Gerő et al., 1961; Tracy et al., 1961; Watts, 1963). Smith (1962) and later Insull and Bartsch (1966) found that the lipids in fibrous plaques resemble those of the main S_f 0 – 12 fraction of low-density lipoproteins in the serum.

The ability of low-density serum lipoproteins to form complexes with various macromolecular polyanions has been described by several investigators. Similarly, glycosaminoglycans isolated from human aorta, being macromolecular polyanions, form complexes with the β-lipoprotein fraction of human serum (Amenta, Waters, 1960; Gerő et al., 1960; Bihari-Varga et al., 1964). The hypothesis that the glycosaminoglycans of the intimal ground substance may in some way render lipids of the blood insoluble and thereby set the stage for the development of the lesions of atherosclerosis underlines the significance of these complexes.

In our previous investigations the chemical composition, stability and specificity of *in vitro* complexes precipitated from human serum and from the

saline extracts of atherosclerotic aorta intimas with aortic glycosaminoglycans have been studied and it was shown that S_f 0—20 β-lipoprotein was the only serum protein fraction to take part in complex formation (Bihari-Varga, 1965; Bihari-Varga, Gerő, 1966; Bihari-Varga, Végh, 1967). In addition Tracy et al. (1965) succeeded in extracting a compound from the aorta which had the same electrophoretic mobility as the *in vitro* complexes previously demonstrated by us.

The object of the present investigations was to prove the existence of glycosaminoglycan-β-lipoprotein complexes, in the aortic tissue.

In a previous communication (Simon et al., 1968) we have reported a derivatographic method suitable for the identification of glycosaminoglycans also in their protein-bound form. When the rate of weight change of glycosaminoglycan-containing samples was plotted as a function of temperature, a characteristic peak was found on the curves at about 240 °C.

The same method was used for the investigation of *in vitro* glycosaminoglycan-β-lipoprotein complexes, of these complexes dissociated by polar organic solvents and of healthy and atherosclerotic aorta intimas without and after delipidation.

Materials and Methods

Isolation and analysis of β-lipoprotein

S_f 0—12 lipoprotein was isolated in the ultracentrifuge from pooled serum samples of hyperlipemic patients by Dr. A. Czuppon in the Laboratory of Research of Chemical Structure of the Hungarian Academy of Sciences. On paper electrophoresis in veronal buffer the lipoprotein preparation migrated as a single component and it contained 16 per cent protein and 78 per cent total lipid. For thermal analysis samples in a freeze-dried form were used.

Isolation, analysis and delipidation of glycosaminoglycan-serum-lipoprotein complexes

A large excess of aortic glycosaminoglycan preparation, prepared as described previously (Bihari-Varga et al., 1964), was added to pooled hyperlipemic sera (5 mg/ml serum) in the presence of 0.02 per cent $CaCl_2$, and the precipitated complex was separated by centrifugation. It was washed twice with 0.02 per cent $CaCl_2$ solution and dried for 3 hours with 50 per cent saturated air at constant flow-rate. The average composition of the samples was 77, 15, and 7 per cent for total lipid, protein and glycosaminoglycan, respectively.

Delipidation of the complexes was performed by extraction in 25 per cent ether in ethanol for 4 days, followed by 50 per cent chloroform in methanol for 3 days, both at room temperature in the dark.

Preparation of aortic intimas

1) Two groups of human aortas were obtained from patients at autopsy within 24 h of death; young, apparently normal aortas and aortas with atherosclerotic intimas. Some atherosclerotic aortas were subdivided according to the types of lesion. Four types of tissue were selected for analysis, these were: *a)* apparently lesion-free intimal areas; *b)* oedematic thickening; *c)* fatty streaks and nodules; *d)* calcified plaques.

After washing the endothelial surface by gentle rubbing under running tap water, the intima was stripped and dried as described for glycosaminoglycan complexes.

2) Arterial specimens from two groups of rabbits were collected, washed and dried immediately after killing the animals; in the first group of rabbits, aged about 6 months, experimental atherosclerosis was produced by feeding 2 g of cholesterol daily for 3 months, while other animals of the same age, kept on standard diet, were used as controls.

Extraction of lipids from aortic samples was performed as described above.

Thermal analysis

The studies were carried out using a F. Paulik – J. Paulik – L. Erdey MOM derivatograph (Paulik et al., 1958). The weight change (TG), rate of weight change (DTG) and enthalpy change (DTA) of the samples have been measured as a function of temperature. The samples were weighed (approximately 100 mg) into a platinum crucible. The heating rate was 10 °C/min up to 900 °C.

Results

Fig. 1 shows typical thermal decomposition curves of the *in vitro* aortic glycosaminoglycan-serum-β-lipoprotein complex. It might be concluded that the incorporation of the lipoprotein moiety into the molecule resulted in an increase in thermostability. Decomposition took place with maximal rate only at 390 °C, according to the DTG curve; the characteristic peak found on the DTG curves of glycosaminoglycan preparation at about 240 °C (Simon et al., 1968), diminished here to an inflexion.

Fig. 2 presents the thermal decomposition curves of β-lipoprotein. It is to be seen that after the departure of 1 per cent adsorbed moisture, decomposition of the sample starts at 150 °C and the maximum rate is at 315 °C. The endotherm peak on the DTA curves at 790 °C is the melting point of sodium chloride, added to the sample during the ultracentrifugal isolation procedure.

The derivatogram of the delipidated *in vitro* glycosaminoglycan-β-lipoprotein complexes is shown in Fig. 3. It is remarkable that after the extraction of lipids the maximum at 270 °C reappears on the DTG curves, which is followed, similarly

to all the previously investigated samples, by a smaller weight change at 330 °C. It is to be seen that the shape of the curves obtained after delipidation resembles that of the curves obtained with chondroitin-4-sulfate-protein complexes (Simon et al., 1968).

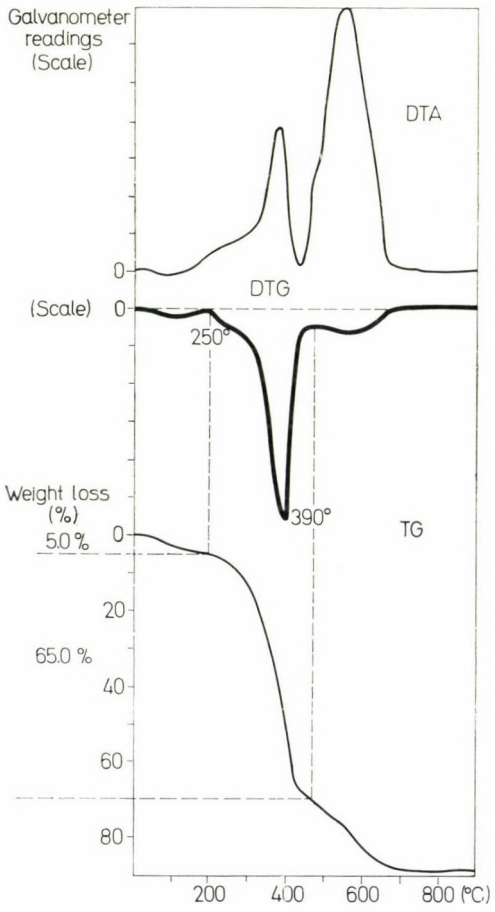

Fig. 1. Aortic glycosaminoglycan-β-lipoprotein complex

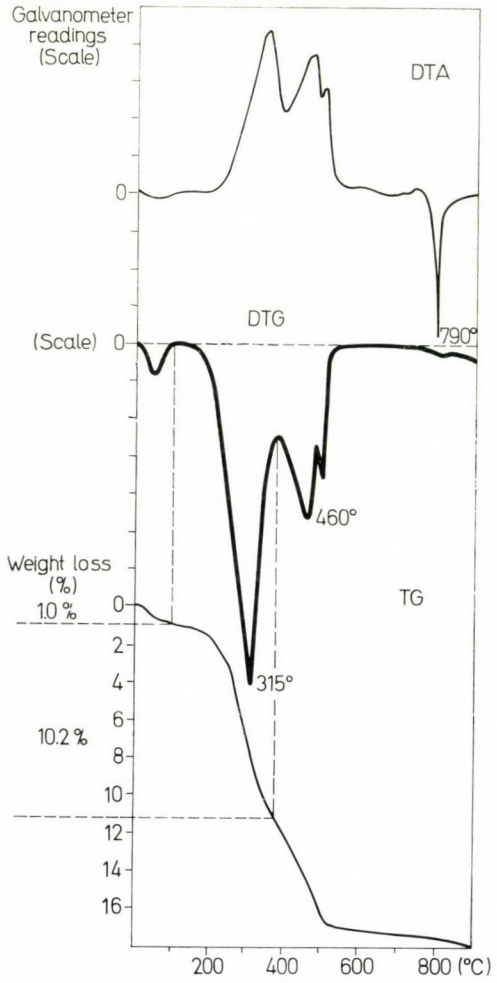

Fig. 2. β-lipoprotein

Derivatogram of young, healthy human aorta intima is presented in Fig. 4. Water content gets lost first, the decomposition starts at about 200 °C. According to the DTG curve, the first process, that reaches its maximum rate at 260 °C, results in 7 per cent weight loss. (This peak was found to be characteristic of the

decomposition of glycosaminoglycans and glycosaminoglycan-protein complexes.) The main reaction, leading to 46 per cent weight loss, occurs at 320 °C with maximum rate.

Fig. 5 shows thermal decomposition curves of atherosclerotic human aorta intima. It can be seen that the DTG maximum previously present in the 240–

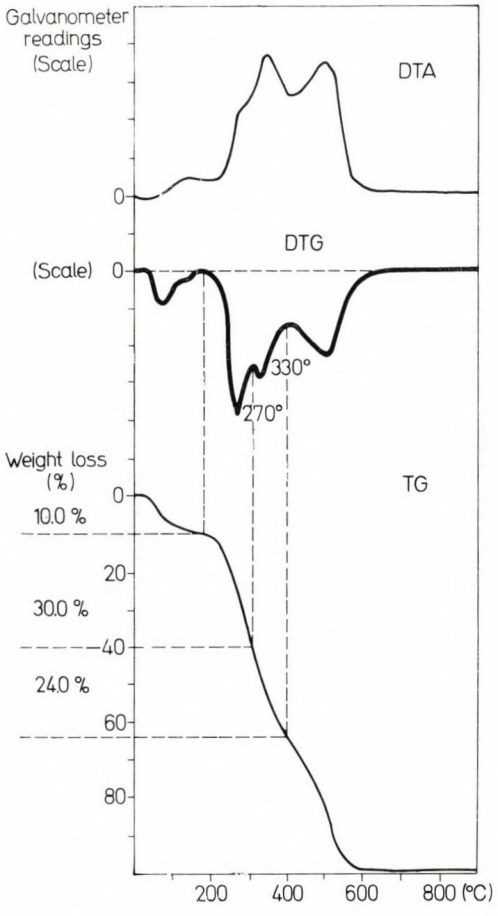

Fig. 3. Delipidated glycosaminoglycan-β-lipoprotein complex

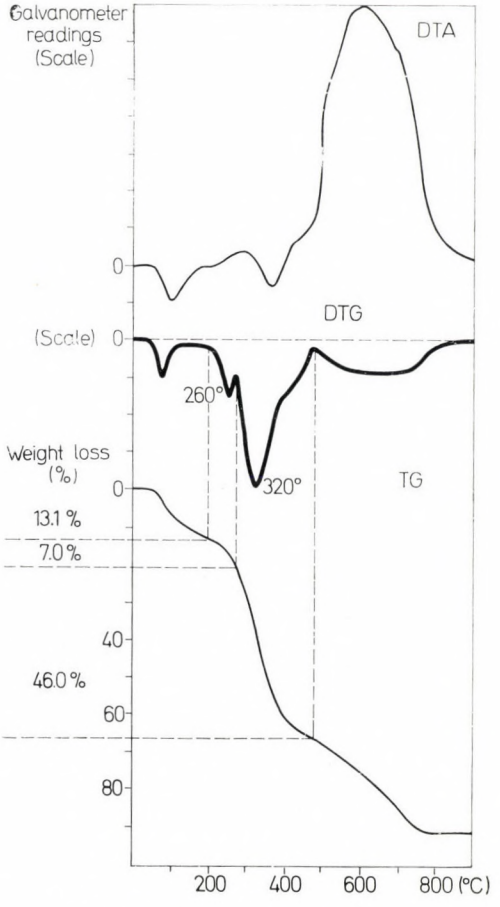

Fig. 4. Healthy human aorta intima

270 °C interval, diminished to an inflexion, in some cases it even disappeared, and only the 320–330 °C DTG maximum is observable, presumably due to the decomposition of structural proteins.

The endotherm enthalpy change between 200–400 °C shown by the DTA curves of both healthy and atherosclerotic aortas indicates that mainly decomposition and the removal of the liberated products take place in this interval.

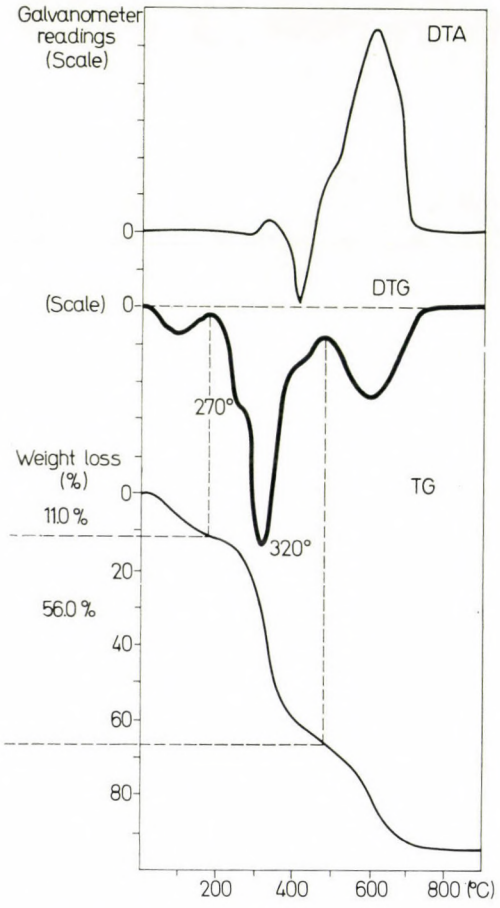

Fig. 5. Atherosclerotic human aorta intima

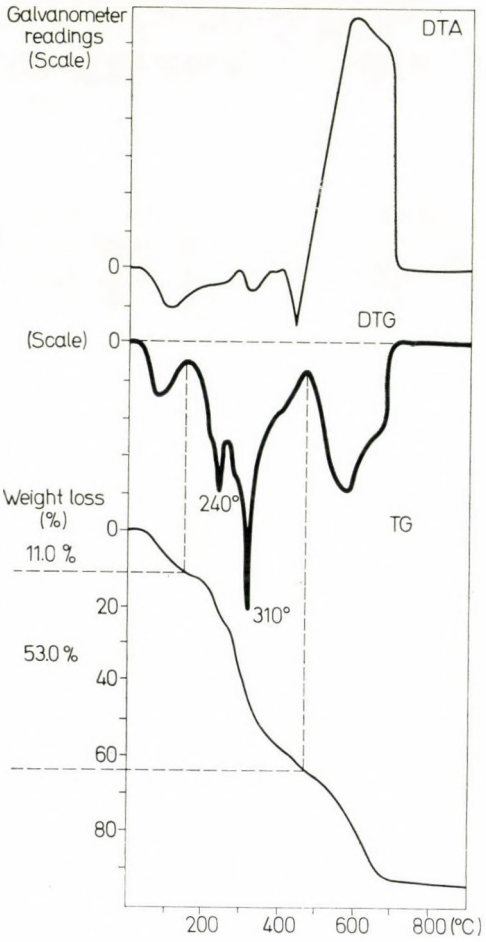

Fig. 6. Delipidated atherosclerotic human aorta intima

In the diagrams obtained with delipidated atherosclerotic human aortas (Fig. 6) it is of importance that lipid extraction resulted in a decrease of thermostability and that the curves resemble those of healthy human intimas.

Fig. 7 shows the derivatograms of different parts of atherosclerotic intimas. On the DTG curve of the apparently lesion-free parts there is a small peak at 240 °C (Fig. 7/a). This decreased to an inflexion in tissue samples with oedematic

thickening (Fig. 7/b) and was hardly visible with fatty streaks and nodules (Fig. 7/c). With calcified plaques (Fig. 7/d) the 240 °C DTG peak was completely missing, however, a double maximum appeared consequently at 320 °C and 370 °C on the DTG curves. It was observed that on the TG curves of calcified samples the weight

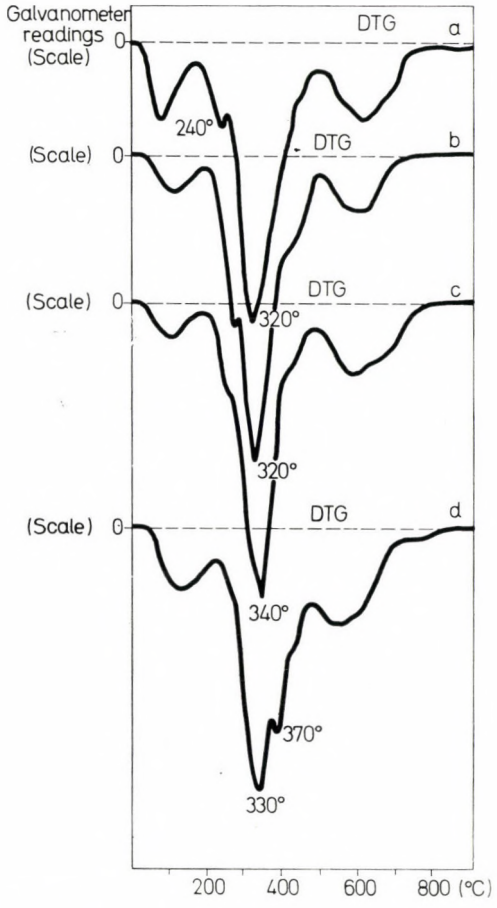

Fig. 7. Different parts of atherosclerotic human aorta intima. *d)* lesion-free areas, *b)* oedematic thickening, *c)* fatty streaks and nodules, *d)* calcified plaques

loss up till 800 °C was 40–45 per cent, thus this tissue contained 50–55 per cent inorganic components. It could be established by X-ray diffraction method that the ash contained tricalcium phosphate and calciumpyrophosphate.

The thermoanalytical decomposition curves of aortas of healthy, young rabbits (Fig. 8/a), of rabbits with cholesterol-induced atherosclerosis (Fig. 8/b) and of the delipidated parallel samples of the latter (Fig. 8/c) are in good agreement with those of the corresponding human tissue samples (see Figs 4, 5 and 6).

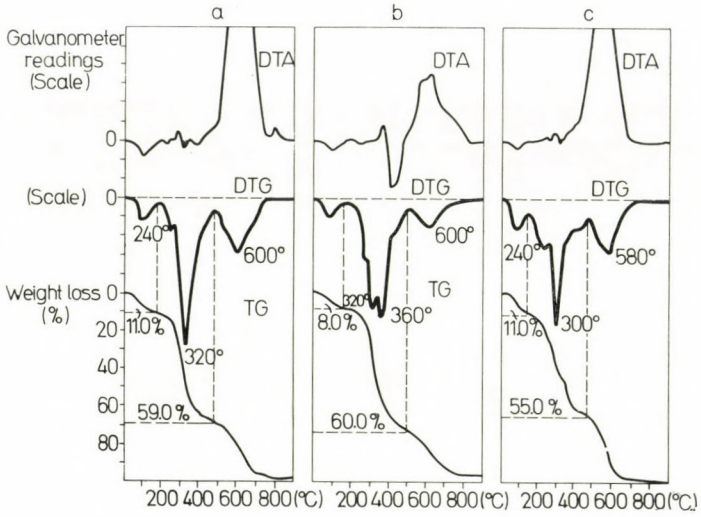

Fig. 8. Rabbit aorta intimas. *a)* healthy aorta intima, *b)* atherosclerotic aorta intima, *c)* delipidated atherosclerotic aorta intima

Discussion

The chemical and biochemical approaches to the study of arterial connective tissue have been attempted to obtain information on the pathomechanism of atherosclerosis. The ways in which vascular glycosaminoglycans may play a role in atherogenesis have been the subject of a number of investigations. Although these approaches have been useful, they are all handicapped by the fact that all the methods used for isolation, separation and identification of glycosaminoglycans involve the denaturation of the tissue and the destruction of the original macromolecular aggregates.

Derivatographic investigation was found to be a suitable method for the study of structural glycosaminoglycans, being based on the analysis of naturally occurring complex macromolecules.

A characteristic peak found at about 240 °C on the DTG curves of a series of glycosaminoglycan preparations was always detectable in healthy aortic intimas. As described (Simon et al., 1968) this peak (and the thermostability of the molecule) was not affected when the glycosaminoglycan was present in the form of a protein complex. This phenomenon might explain the appearance of the characteristic glycosaminoglycan-peak on the derivatogram of aortic tissues, in which the glycosaminoglycan is bound to the structural proteins. The possibility that some other components of the aortic tissue (e.g. proteins, lipids) may also produce a DTG peak in the 240 °C area, can be excluded, as the decomposition of these compounds of higher thermostability takes place at more elevated temperatures.

On the other hand, when glycosaminoglycans were converted *in vitro* into a glycosaminoglycan-lipoprotein complex, an increase in thermostability could be established, resulting in a significant decrease (in some cases even a lack) of the 240 °C DTG maximum. In these complexes (and also in pure β-lipoprotein) thermal decomposition took place with maximal rate far above 300 °C. The suggestion that the disappearance of the peak was a consequence of complex formation was supported by the effect of delipidation; on the curves obtained with samples in which the lipoprotein complex was dissociated by extraction with organic solvents, the peak became again detectable. Thermal decomposition curves of atherosclerotic intimas (both human and experimental) in the 240 °C area resembled those of the *in vitro* glycosaminoglycan-lipoprotein complexes. As an effect of delipidation the glycosaminoglycan-peak reappeared also in this case and the derivatograms of delipidated atherosclerotic aorta became similar to that of the healthy tissue.

A correlation was established between the gradual decrease of the DTG peak and the evolution of the atherosclerotic lesions. (It has to be mentioned that in the present experimental conditions, the exact determination of the glycosaminoglycan-β-lipoprotein complexes in the aorta cannot be performed and so the presence of small quantities of β-lipoprotein in the healthy intima cannot be excluded.)

The results reported above seem to provide evidence for the *in vivo* existence of glycosaminoglycan-β-lipoprotein complexes in the atherosclerotic intima, underlining the role of glycosaminoglycans in the mechanism of lipid deposition into the vessel wall during the process of atherogenesis.

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Kinetics of the Reactions Catalyzed by D-Glyceraldehyde-3-phosphate Dehydrogenase

IV. Activation by Excess Substrate and Inhibition by Product

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Saturation curves by both NAD and D-glyceraldehyde-3-phosphate of D-glyceraldehyde-3-phosphate dehydrogenase show anomalies in the presence of phosphate as third substrate. The excess of NAD inhibits, that of D-glyceraldehyde-3-phosphate activates the enzyme.

The kinetic analysis of enzymological data shows that there are distinct substrate and product binding sites on the enzyme, further that substrate and product may be bound to the binding site of each other. The substrate bound to the product binding site influences the rate of the breakdown of substrate bound to the substrate binding site.

NADH inhibits D-glyceraldehyde-3-phosphate oxidation competitively with NAD, however, this is a parabolic competitive inhibition. Glyceric acid-3-phosphate, the analogue of the end-product glyceric acid-1,3-diphosphate also inhibits D-glyceraldehyde-3-phosphate dehydrogenase and this inhibition is a parabolic mixed type with D-glyceraldehyde-3-phosphate.

In previous works we dealt with the kinetics and mechanism of GAPD* using GAP (Keleti, Telegdi, 1959; Keleti, Batke, 1965, 1967) and GA (Keleti, 1965, 1966) as substrate. In these works phosphate was used as third substrate and all substrates were used in optimum concentration. The results show the random binding of NAD and GAP to the enzyme and also that the kinetics follow the rapid equilibrium assumption, where phosphorolysis of oxidized substrate is the rate limiting step.

In the present paper we investigate the effect of excess substrate and of end-product, on the rate of the reaction. NADH or excess NAD inhibits while the excess of GAP activates the enzyme. The kinetic analysis of inhibition and activation by substrates and products show that substrates and products may also be bound to the binding site of each other.

* Abbreviations used: GAPD: D-glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD oxidoreductase, phosphorylating, EC. 1. 2. 1. 12); GAP: D-glyceraldehyde-3-phosphate; GA: D-glyceraldehyde; GS: glyceric acid; GSP: glyceric acid-3-phosphate; GSPP: glyceric acid-1,3-diphosphate; NAD and NADH: oxidized and reduced form of nicotinamide-adenin-dinucleotide, respectively; LDH: lactic dehydrogenase (L-lactate:NAD oxidoreductase, EC. 1. 1.1.27).

Materials and Methods

Five times recrystallized swine muscle GAPD (Elődi, Szörényi, 1956) was used. The molecular weight of 140 000 (Elődi, 1958) was used in the calculations.

GAP was prepared from fructose-1,6-diphosphate (Reanal) according to Szewczuk et al. (1961).

NAD (Boehringer or Reanal) and NADH (Reanal) was purified on DEAE cellulose (Silverstein, 1965).

GSP, GS and GA were Fluka preparations.

$^{14}C-GS$ was Reanal preparation of 0.837 mCi/mM specific activity.

The other chemicals were commercial preparations of reagent grade.

Enzymic activity was determined by Warburg's optical test at 340 mμ. Protein concentration was measured spectrophotometrically at 280 mμ. Enzymic activity was assayed in 0.1 M glycine buffer, pH 7.0 to 9.0, in cuvettes of 1 cm light path, at room temperature. GAPD concentration of 7.15×10^{-9} M was used. Calculations were made from the increase in optical density obtained in the first five seconds after the addition of enzyme. The initial velocity v_0, is given in M NADH formed in five seconds.

Measurements were carried out in a Hilger UVISPEK spectrophotometer and in an Opton PMQ II spectrophotometer.

Results

The NAD saturation curve of GAPD shows anomalies in the presence of GAP and phosphate, since no hyperbola was obtained (Fig. 1). The optimum concentration of NAD is about 4×10^{-4} M and inhibition by excess substrate approaches a constant level (at above 10^{-2} M NAD).

NADH inhibits GAP oxidation and this inhibition is competitive with NAD (Fig. 2a), however, the Dixon plot (i.e. the reciprocal of reaction velocity plotted in the function of inhibitor concentration) is non-linear (Fig. 2b).

The GAP saturation curve of GAPD also shows anomalies (Fig. 3). The excess of substrate activates the enzyme. Similar activation was also observed by excess GA (Keleti, 1966).

GSP, the analogue of GSPP inhibits GAPD, with respect to GAP a mixed type inhibition is obtained (Fig 4a). The Dixon-plot is again non-linear (Fig. 4b).

To obtain further information on the binding of end-product to the enzyme we studied the reverse reaction of GA oxidation – which proceeds without phosphate also (Keleti, 1966). In the present experiments we find that GAPD does not catalyze the reduction of GS*. We have further found with $^{14}C-GS$ that

* If the GAPD preparation is contaminated with LDH, an apparent reduction of GS will be obtained, since GS is a substrate of LDH. There is a parallel loss of pyruvate reduction, lactate oxidation and GS reduction as GAPD is purified from contaminating LDH.

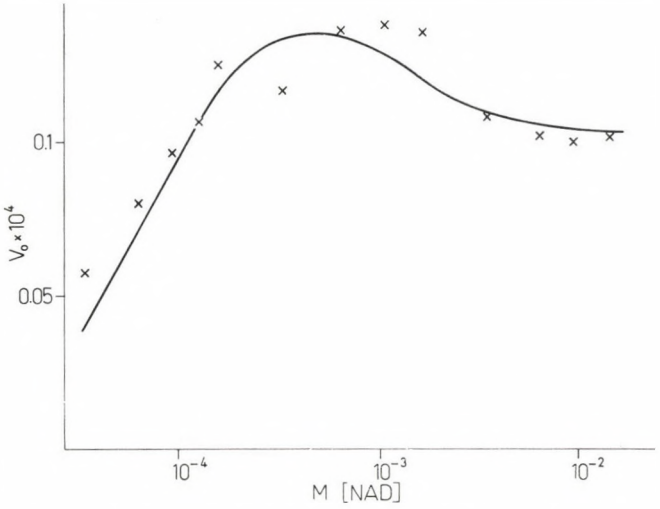

Fig. 1. The NAD saturation curve of GAPD, if GAP and phosphate concentrations are constant. GAP: 1.38×10^{-3} M; phosphate: 1×10^{-2} M; GAPD: 7.15×10^{-9} M. In 0.1 M glycine buffer, pH 8.5. *x*, experimental points. ———, theoretical curve, calculated from equation (19) with the use of the constants in Table 1

GAPD – GS binary complex is not formed either in the presence or in the absence of NAD or NADH.

If GA oxidation proceeds in the presence of phosphate ion, glyceric acid-1-phosphate is formed instead of GS. If the reaction mixture which contains GAPD, GA, NAD, and phosphate after the reaction has reached the equilibrium is acidified below pH 6.5, the reverse reaction becomes measurable, and the optical density at 340 mμ decreases since glyceric acid-1-phosphate reacts with NADH. The pH optimum of this reaction is at pH 5.5. However, below pH 5.3 it is not possible to measure the reverse reaction because of the spontaneous decomposition of NADH at these pH values.

GAPD does not catalyze the reaction between acetate and NADH. However, GAPD catalyzes the hydrolysis of acetic anhydride and during this reaction an S-acetyl-GAPD intermediate (Polgár, 1964) is formed.

If we prepare S-acetyl-GAPD by treating the enzyme with acetic anhydride, this intermediate will react with NADH at pH 6.0 to 6.5.

Theoretical

We may assume that: 1. the enzyme is saturated by the substrate or by coenzyme. (We defined the following symbols: E = E – GAP or E' = E – NAD); 2. the rate limiting step in the mechanism of GAPD action is the phosphorolysis

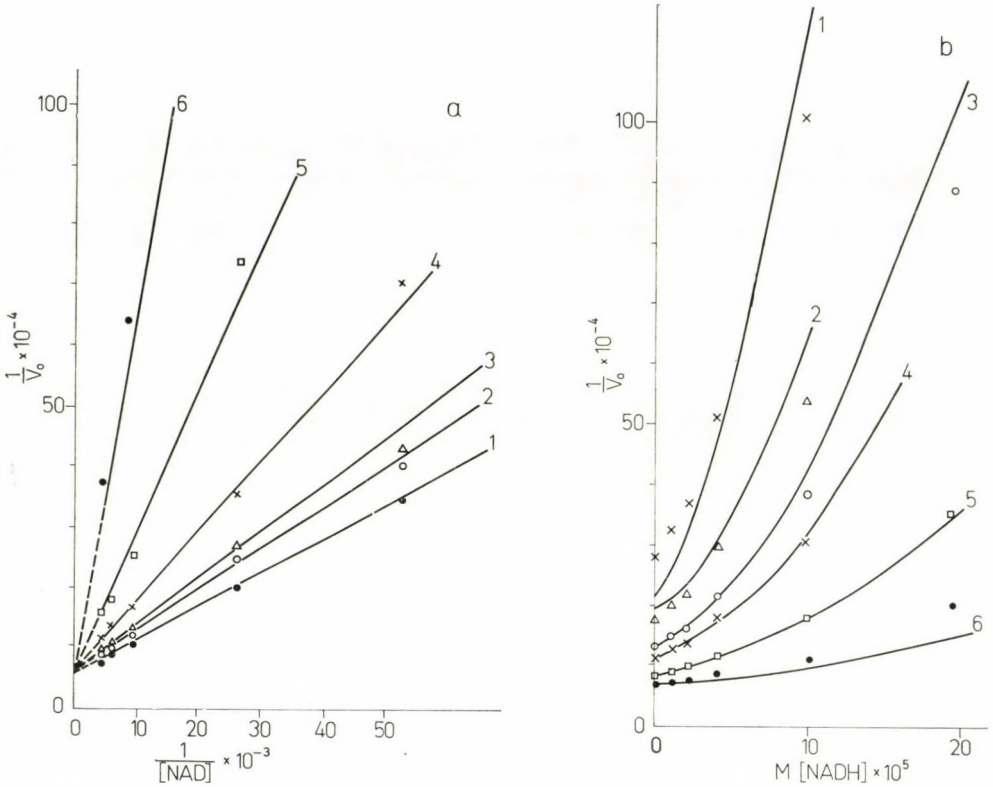

Fig. 2. The inhibition of GAPD with NADH. *a)* The Lineweaver-Burk plot. GAP: 1×10^{-3} M; phosphate: 1×10^{-2} M; GAPD: 7.15×10^{-9} M. NADH = 1: 0 M; 2: 0.96×10^{-5} M; 3: 1.93×10^{-5} M; 4: 3.86×10^{-5} M; 5: 9.65×10^{-5} M; 6: 19.3×10^{-5} M. In 0.1 M glycine buffer, pH 8.5. *b)* The Dixon plot. Conditions as in the legend to Fig. 2a. NAD = 1: 0.25×10^{-4} M; 2: 0.5×10^{-4} M; 3: 0.75×10^{-4} M; 4: 1×10^{-4} M; 5: 2×10^{-4} M; 6: 4×10^{-4} M. □, △, ○, ●, *x*: experimental points. ———: theoretical curves calculated from equation (21) with the use of the constants in Tables 1 and 2

of oxidized substrate (Keleti, Telegdi, 1959; Keleti, Batke, 1965, 1967); 3. there are distinct substrate and product binding sites and both substrate and product can be bound to both binding sites. In this case the following elementary steps may be assumed in the catalytic process:

$$(1)\ \bar{E} + NAD \rightleftharpoons \bar{E}\text{-}NAD \rightarrow k_1 \qquad K_1 = [\bar{E}][NAD]/[\bar{E}\text{-}NAD]$$

$$(2)\ \bar{E} + NAD \rightleftharpoons NAD\text{-}\bar{E} \qquad K_2 = [\bar{E}][NAD]/[NAD\text{-}\bar{E}]$$

$$\left.\begin{array}{l}(3)\ E\text{-}NAD + NAD \rightleftharpoons NAD\text{-}\bar{E}\text{-}NAD \\ (4)\ NAD\text{-}\bar{E} + NAD \rightleftharpoons NAD\text{-}E\text{-}NAD\end{array}\right\} \xrightarrow{k_2} \qquad K_3 = \frac{[E\text{-}NAD][NAD]}{[NAD\text{-}\bar{E}\text{-}NAD]}$$

$$K_4 = \frac{[NAD\text{-}\bar{E}]\,[NAD]}{[NAD\text{-}\bar{E}\text{-}NAD]}$$

$$(5)\ \bar{E} + NADH \rightleftharpoons \bar{E}\text{-}NADH \qquad K_5 = \frac{[\bar{E}][NADH]}{[E\text{-}NADH]}$$

$$(6)\ \bar{E} + NADH \rightleftharpoons NADH\text{-}\bar{E} \qquad K_6 = \frac{[\bar{E}][NADH]}{[NADH\text{-}E]}$$

$$(7)\ \bar{E}\text{-}NADH + NADH \rightleftharpoons NADH\text{-}\bar{E}\text{-}NADH \qquad K_7 = \frac{[\bar{E}\text{-}NADH][NADH]}{[NADH\text{-}E\text{-}NADH]}$$

$$(8)\ NADH\text{-}\bar{E} + NADH \rightleftharpoons NADH\text{-}\bar{E}\text{-}NADH \qquad K_8 = \frac{[NADH\text{-}\bar{E}][NADH]}{[NADH\text{-}E\text{-}NADH]}$$

$$(9)\ \bar{E}\text{-}NAD + NADH \rightleftharpoons NADH\text{-}\bar{E}\text{-}NAD \qquad K_9 = \frac{[\bar{E}\text{-}NAD][NADH]}{[NADH\text{-}E\text{-}NAD]}$$

$$(10)\ NADH\text{-}E + NAD \rightleftharpoons NADH\text{-}\bar{E}\text{-}NAD \xrightarrow{k_3} \qquad K_{10} = \frac{[NADH\text{-}\bar{E}][NAD]}{[NADH\text{-}\bar{E}\text{-}NAD]}$$

$$(11)\ NAD\text{-}\bar{E} + NADH \rightleftharpoons NAD\text{-}\bar{E}\text{-}NADH \qquad K_{11} = \frac{[NAD\text{-}\bar{E}][NADH]}{[NAD\text{-}\bar{E}\text{-}NADH]}$$

$$(12)\ \bar{E}\text{-}NADH + NAD \rightleftharpoons NAD\text{-}\bar{E}\text{-}NADH \qquad K_{12} = \frac{[\bar{E}\text{-}NADH][NAD]}{[NAD\text{-}\bar{E}\text{-}NADH]}$$

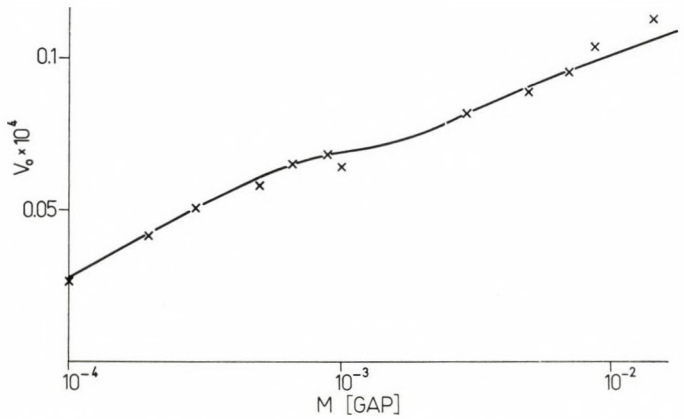

Fig. 3. The GAP saturation curve of GAPD, if NAD and phosphate concentrations are constant. NAD: 1.48×10^{-3} M; phosphate: 1×10^{-2} M; GAPD: 7.15×10^{-9} M. In 0.1 M glycine buffer, pH 7.0. We used this pH since at lower pH values as the pH optimum (pH 8.5) the activatory effect of excess GAP was more pronounced than at the pH optimum. *x:* experimental points. ———: theoretical curve, calculated from equation (19) with the use of the constants in Table 1

where binding to the substrate binding site or to the product binding site is indicated by symbols written to the right or to the left of E, respectively. In the forward reaction only the substrate binding site is catalytically active. If the enzyme is

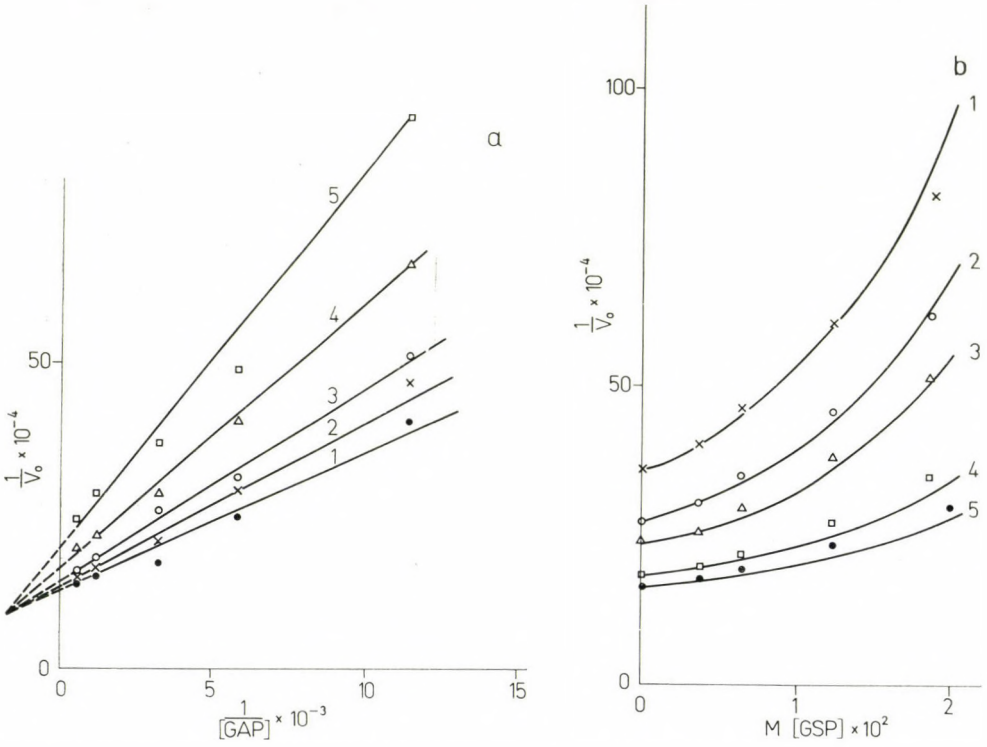

Fig. 4. Inhibition of GAPD with GSP. *a)* Lineweaver-Burk plot. NAD: 1.7×10^{-3} M; phosphate: 1×10^{-2} M; GAPD: 7.15×10^{-9} M; GSP = 1: 0 M; 2: 3.12×10^{-3} M; 3: 6.25×10^{-3} M; 4: 12.5×10^{-3} M; 5: 18.75×10^{-3} M. In 0.1 M glycine buffer, pH 7.2. *b)* Dixon plot. Conditions as in the legend to Fig. 4a. GAP = 1: 1×10^{-4} M; 2: 1.5×10^{-4} M; 3: 2×10^{-4} M; 4: 4×10^{-4} M; 5: 6×10^{-4} M. □, △, ●, ○, *x:* experimental points. ———: theoretical curves, calculated from equation (21) with the use of the constants in Tables 1 and 2

saturated with NAD, 12 similar equations may be written with GAP and GSP, writing $\bar{E}'$ (= E-NAD) instead of $\bar{E}$ (= E-GAP).

It is to be noted that:

(13) $$K_1K_3 = K_2K_4$$

(14) $$K_5K_7 = K_6K_8$$

(15) $$K_1K_9 = K_{10}K_6$$

(16) $$K_2K_{11} = K_{12}K_5$$

If kinetically important binary, ternary and quaternary complexes with phosphate are not formed during the reaction (Keleti, Batke, 1965):

$$(17)\quad [\bar{E}]_T = [\bar{E}] + [\bar{E}\text{-NAD}] + [\text{NAD-}\bar{E}] + [\text{NAD-}\bar{E}\text{-NAD}] + [\text{E-NADH}] + [\text{NADH-}\bar{E}] + [\text{NADH-}\bar{E}\text{-NADH}] + [\text{NAD-E-NADH}] + [\text{NADH-E-NAD}]$$

The equation of velocity:

$$(18)\quad v = k_1[\bar{E}\text{-NAD}] + k_2[\text{NAD-E-NAD}] + k_3[\text{NADH-E-NAD}] = (k_1[S]/K_1 + k_2[S]^2/K_1K_3 + k_3[S][P]/K_1K_9)[E]_T/\{1 + [S](1/K_1 + 1/K_2) + [S]^2/K_1K_3 + [P](1/K_5 + 1/K_6) + [P]^2/K_5K_7 + [P][S](1/K_1K_9 + 1/K_2K_{11})\}$$

where [S] = substrate and [P] = product.

In the case [P] = 0, the initial velocity is given by:

$$(19)\quad v_0 = (k_1[S]/K_1 + k_2[S]^2/K_1K_3)[E]_T/\{1 + [S](1/K_1 + 1/K_2) + [S]^2/K_1K_3\}$$

From the experimental values in Fig. 1 and Fig. 3 the numerical value of all constants may be determined (Table 1) since equation (19) gives both inhibition and activation depending on the value of the following inequality:

$$(20)\quad (k_1K_3 + k_2\sqrt{K_1K_3})/[2\sqrt{K_1K_3} + K_3(1 + K_1/K_2)] \gtrless k_2 \quad \begin{matrix}\text{inhibition}\\ \text{activation}\end{matrix}$$

Table 1

Kinetic constants of equation (19) determined by varying the concentration of NAD, and GAP, respectively

Constant	Varying the concentration of NAD	Varying the concentration of GAP
K_1	0.7 M*	1.4 M*
K_2	2×10^{-4} M	1.74×10^{-4} M
K_3	6×10^{-8} M	1.46×10^{-6} M
K_4	2.1×10^{-4} M	1.18×10^{-2} M
k_1	9.65×10^{-2} (5 sec)$^{-1}$	6.3×10^{-2} (5 sec)$^{-1}$
k_2	1×10^{-5} (5 sec)$^{-1}$	1.3×10^{-5} (5 sec)$^{-1}$

* Values measured independently (Keleti, Batke, 1965).

Equation (18) is valid in the general case, including initial velocity in the presence of added product (i.e. $[P] \neq 0$). However, Fig. 2b and Fig. 4b show that the reciprocal of reaction velocity plotted in the function of inhibitor concentration (the Dixon plot) gives a parabola (cf. Cleland 1963, 1967). Equation (18) gives parabola if and only if $k_3 = 0$, that is if the binding of the product to the product binding site inactivates the enzyme.

In this case:

$$(21)\quad 1/v = \{1 + [S](1/K_1 + 1/K_2) + [S]^2/K_1K_3 + [1/K_5 + 1/K_6 + [S](1/K_1K_9 + 1/K_2K_{11})][P] + [P]^2/K_5K_7\}/(k_1[S]/K_1 + k_2[S]^2/K_1K_3)[E]_1$$

Let us introduce the notations:

$$(22)\quad \alpha = 1/K_5 + 1/K_6 + [S](1/K_1K_9 + 1/K_2K_{11}) \quad \text{and}$$

$$(23)\quad \beta = 1/K_5K_7$$

Since K_1, K_2, K_3, K_4, k_1 and k_2 are obtained from Table 1, the values of α and β can be determined for both NADH and GSP (Table 2).

Table 2

Kinetic constants of equation (21) applied to inhibition with NADH and GSP

Inhibition with NADH		Concentration of NAD in $M \times 10^4$	Inhibition with GSP		Concentration of GAP in $M \times 10^4$
$\alpha \times 10^{-4}$	$\beta \times 10^{-8}$		α	$\beta \times 10^{-3}$	
1.74	1.75	0.25	60	2.72	1
1.03	2.12	0.5	78	1.95	1.5
2.54	1.57	0.5	58.5	4.1	2
1.89	1.63	1.0	58	5.3	4
1.87	1.52	1.0			
1.58	1.76	2.0	145	1.6	5
1.12	2.48	2.0			

The data of Table 2 show that the value of α is independent of [S]. This is possible if and only if:

$$(24)\quad 1/K_1K_9 + 1/K_2K_{11} = 0.$$

Since $1/K_1 \neq 0$ and $1/K_2 \neq 0$ it follows that $1/K_9 = 1/K_{11} = 0$. In this case, it further follows from equations (15) and (16) that $1/K_{10} = 1/K_{12} = 0$, that is the elementary steps (9)–(12) do not function, which means that mixed substrate-enzyme-product complexes are not formed.

From the value of α and β it is not possible to determine the values of K_5, K_6, K_7 and K_8 except one of these constants can be determined by an independent method, since we only have three equations (14), (22), (23) and four unknowns.

Discussion

The anomalies in saturation curve for NAD and GAP as well as the parabolic Dixon plot of NADH and GSP inhibition can be described with equilibriums (1) to (8) if we assume that there are distinct substrate and product binding sites

and that the substrate can be bound to both binding sites. However, the kinetic data show that mixed substrate-enzyme-product complexes (e.g. NADH-$\bar{E}$-NAD) are not formed and binding of the substrate to the substrate binding site will only yield product in the forward reaction.

The spectrofluorimetric study of enzyme complexes shows that ternary complexes of the GAP-GAPD-NADH type are formed – that is ternary complexes which involve one of the substrates and the product of the other substrate (Smith, 1966).

NADH and GSP inhibit the activity of GAPD. The Dixon plot of these inhibitions are parabolic. Purely competitive, purely non-competitive or mixed type of inhibition should yield a linear Dixon plot. Partially competitive, partially non-competitive or apparently mixed types of inhibition should yield a hyperbolic Dixon plot (Dixon, Webb, 1964; Keleti, Telegdi, 1966).

A parabolic Dixon plot is consistent with the assumption that:

1. The inhibitor may be bound to both substrate and product binding sites, and 2. mixed substrate-enzyme-product complexes are not formed.

The parabolic mixed type of GSP inhibition also shows that the substrate and product binding sites are different. However, this statement is equivocal since the experiments with GS and acetate show that carboxyl groups which does not contain energy-rich bond cannot be bound to the enzyme at the product binding site. (On the other hand, in agreement with the experiments glyceric-acid-1-phosphate or acetic anhydride is a substrate of GAPD in the reverse direction because of their energy-rich anhydride bond which makes it able to form S-acetyl complex with the enzyme.) Since it was demonstrated that the binding of one substrate influences the binding of the other (Keleti, Batke, 1965) it may be assumed that not only the dissociation constant is affected* but also the number of bound coenzyme. It is known that GAPD is formed from 4 identical subunits (Harris, Perham, 1965; Harrington, Karr, 1965). If substrate is bound to the enzyme, the coenzyme may be bound not only to the coenzyme binding group (one per subunit) but also to the reduced coenzyme binding group. This assumption would be consistent with the quadratic relationship between reaction velocity and substrate concentration. The isomerization of the enzyme may result in a quadratic rate equation in the case of steady-state kinetics only (Keleti, 1967, 1968). However, the reaction catalyzed by GAPD follows the rapid equilibrium assumption (Keleti, Telegdi, 1959; Keleti, Batke, 1965, 1967) and this can only yield a hyperbolic rate equation, even in the case of the isomerization of the enzyme (Keleti, 1967).

* It is to be noted that:

$K_1 = [\bar{E}][NAD]/[\bar{E}—NAD] = 0.7$

$K_4 = [NAD—\bar{E}][NAD]/[NAD—\bar{E}—NAD] = 2.1 \times 10^{-2}$

$K_2 = [E][NAD]/[NAD—\bar{E}] = 2 \times 10^{-4}$

$K_3 = [\bar{E}—NAD]\ [NAD]/[NAD—\bar{E}—NAD] = 6 \times 10^{-8}$

[cf. equations (1)–(4) and Table 1]

$K_1/K_4 = 3.3 \times 10^3$ and $K_2/K_3 = 3.3 \times 10^3$ [cf. equation (13)]

It is to be noted that similar results obtained only with kinetic methods give the possibility of some assumptions. However, it is necessary to obtain evidences with other independent methods to validate these assumptions.

Appendix

Both curve of Fig. 1 and Fig. 3 may be resolved into two curves (Fig. 5), i.e. $v = v_1 + v_2$ (see equation (18) if $v_3 = 0$).

$$v_1 = k_1K_3[S][E]_T/[K_1K_3 + (K_3 + K_4)[S] + [S]^2] \tag{25}$$

$$v_2 = k_2[S]^2[E]_T/[K_1K_3 + (K_3 + K_4)[S] + [S]^2] \tag{26}$$

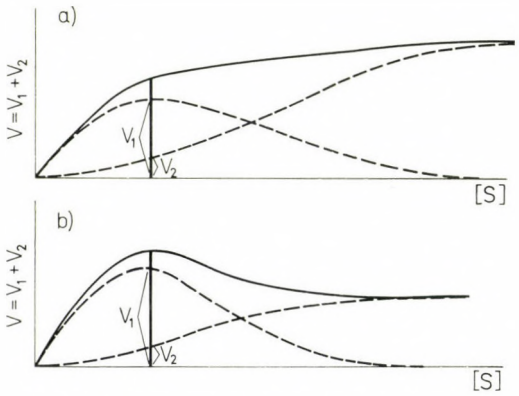

Fig. 5. Resolution of inhibition or activation by excess substrate into two components. *a)* Fig. 3. Resolved into two components. *b)* Fig. 1. Resolved into two components

The maximum of v_1 is where $dv_1/d[S] = 0$, i.e. if

$$[S] = \sqrt{K_1K_3}$$

In this case:

$$v_m = (k_1K_3 + k_2\sqrt{K_1K_3})[\bar{E}]_T/[2\sqrt{K_1K_3} + K_3(1 + K_1/K_2)] \tag{27}$$

where v_m is the value of $v = v_1 + v_2$ at the maximum position of v_1, i.e. if $[S] = \sqrt{K_1K_3}$.

There is inhibition if v_m is greater than the maximum of v_2 and there is activation if this inequality is reversed (see Fig. 5). Since v_2 reaches its maximum position if $[S] \to \infty$ and in this case $v_2 = k_2[\bar{E}]_T$ and $v_1 \to 0$:

There is inhibition if $v_m > k_2[\bar{E}]_T$ and activation if $v_m < k_2[\bar{E}]_T$ [see equation (20)].

There is activation or inhibition if:
$k_1 \neq k_2 \neq 0$ depending on the numerical values of different constants. If:

$$k_2(K_1 + K_2)/k_1K_2 > 1 \quad \text{and} \quad k_1(K_1 + K_2)/K_1K_2 > k_2/K_3$$

we have a curve of the type shown in Fig. 3. If the latter inequality is reversed, we have an activation of the sigmoid type. If the first inequality is reversed, we have inhibition (Botts, 1958).

There is only activation if:

$$k_1 = k_2 \neq 0. \text{ If } (K_1 + K_2)/K_1K_2 > 1/K_3$$

we have a curve of the type shown in Fig. 3. If this inequality is reversed, the curve will be sigmoid.

There can only be a sigmoid type of activation if: $k_1 = 0$ and $k_2 \neq 0$.

There is only inhibition if: $k_1 \neq 0$ and $k_2 = 0$.

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Conversion of the Serine Residue at the Active Site of Alcalase to a Cysteine Side Chain

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Alcalase, a bacterial protease, was purified by ion exchange chromatography, and its concentration was determined by titrating the active site of the enzyme with *N-trans*-cinnamoylimidazole. Thiol-alcalase was prepared similarly to thiol-subtilisin (Polgár, Bender, 1966). The serine residue at the active site was reacted with phenylmethylsulfonyl fluoride, then the phenyl-methylsulfonyl group was displaced by thiolacetate ion. In contrast to subtilisin, the rate of this displacement reaction is not significantly increased at acidic pH, but a marked rate enhancement is observed at elevated temperature. The absorption maximum of the cinnamoyl-thiol-alcalase prepared in this way is characteristic of a thiol ester. Although the kinetic constants of alcalase are higher than that of subtilisin and alcalase is more stable in alkaline medium, the transformation of the two enzymes into the corresponding thiol-derivative results in similar alterations in catalytic properties and in chromatographic behaviour.

Replacement of the reactive serine side chain at the active site of subtilisin by a cysteine residue results in the formation of thiol-subtilisin (Polgár, Bender, 1966; Neet, Koshland, 1966; Polgár, Bender, 1967; Polgár, Bender, 1969). This thiol-enzyme retains enzymatic activity towards NPA* substrate, although the rate constant of the hydrolysis is 30 times lower than that found with subtilisin. The decrease of activity toward peptides and simple esters is considerably higher. Since by revealing the relationship between a serine enzyme and its thiol-derivative one may obtain an insight into the mechanism of action of the active site, it is important to carry out the hydroxyl-sulfhydryl transformation with a number of enzymes and to compare the results to those found with subtilisin. Alcalase, a proteolytic enzyme isolated from B. subtilis, appears to be suitable for this purpose. It contains no disulfide bridges which would interfere with the modification reaction. The activity of alcalase towards some substrates is significantly higher than the activity of subtilisin (Barel, Glazer, 1968) and the amino acid sequence of the two proteases is different at more than 80 positions (Smith et al., 1968). The stability of alcalase is considerably higher both at high temperature and at alkaline pH. In this paper we describe the preparation of thiol-alcalase and discuss some of the properties of the modified enzyme.

* Abbreviations: NPA, *p*-nitrophenyl acetate; NZG, *p*-nitrophenyl N-benzyloxycarbonyl-glycinate; PMB, *p*-mercuribenzoate; PMS, phenylmethylsulfonyl; CMC, carboxymethyl cellulose.

Materials and Methods

Alcalase was a product of the Novo Pharmaceutical Co. Ltd. NPA was synthetized from *p*-nitrophenol and acetic anhydride. It was recrystallized from alcohol-water and the product melted at 77.5 – 78.0 °C. NZG was purchased from Cyclo Chemical Co.; PMSF-1'-^{14}C from Calbiochem, Inc.; PMB from the British Drug Houses, Ltd., *N-trans*-cinnamoylimidazole from Sigma Chemical Co.; thiolacetic acid from Dr Theodor Schuchardt GmbH. Whatman CM11 carboxymethyl cellulose was used for ion exchange chromatography.

Ion exchange chromatography was performed as described elsewhere (Polgár, Bender, 1969). Radioactivity was measured on a Tri-Carb liquid scintillation counter. For spectrophotometric titration an Opton spectrofluorimeter was used.

The operational concentration of alcalase was determined by a direct spectrophotometric titration of the active site of the enzyme with *N-trans*-cinnamoylimidazole. 0.1 ml of titrant in acetonitrile (approx. 5×10^{-3} M) was added to 2.8 ml of 0.1 M acetate buffer, pH 5.0. The slow spontaneous hydrolysis was followed for 5 minutes. Thereafter, 0.1 ml of the enzyme solution was added (zero time). The initial "burst" in the decrease of absorption of *N-trans*-cinnamoylimidazole upon addition of the enzyme was used for the calculation of the concentration of alcalase. The decrease in the absorption of the titrant was followed at 335 mμ using a molecular extinction coefficient for *N-trans*-cinnamoylimidazole $\varepsilon_{335} = 9.37 \times 10^3$. Cinnamic acid and imidazole do not absorb under the same conditions and the absorption of cinnamoyl-alcalase is very low, ε_{335} is about 0.17×10^3. The molarity of alcalase in the cell (M) was calculated from the following equation:

$$M = \frac{(v_i/v_f)\, A_i + A_e - A_f}{9.20 \times 10^3}$$

where v_i is the initial volume before the addition of the enzyme, v_f is the final volume after the addition of the enzyme, A_i and A_f are the absorbancies extrapolated to time 0 from the spontaneous hydrolysis of the substrate and from the enzymatic steady-state turnover, respectively. A_e (which is determined separately) accounts for the absorbance of the enzyme in the final volume of the reaction mixture.

Results

Ion exchange chromatography of alcalase. The commercial preparation was purified on a CMC column equilibrated with 0.01 M phosphate buffer, pH 6.5 by a linear gradient elution using 0.1 M phosphate buffer, pH 6.5. The same experimental conditions were previously employed to isolate pure subtilisin (Polgár, Bender, 1969). Alcalase exhibited a similar chromatographic pattern to that found with subtilisin: 18 per cent of the absorption at 280 mμ applied to the CMC passed through the column and 82 per cent of it could be eluted by increasing

the concentration of the phosphate buffer. This latter peak proved to be homogeneous alcalase when tested with the NPA substrate. The first peak contained some coloured material and denatured protein. Table 1 shows that 58 per cent of

Table 1

Purification of alcalase by ion exchange chromatography

Total absorption at 280 mμ of	Per cent
solution of the commercial preparation	100
protein fraction recovered from the Sephadex column	76
the fraction not bound to CMC	13
alcalase eluted by increasing buffer concentration	58

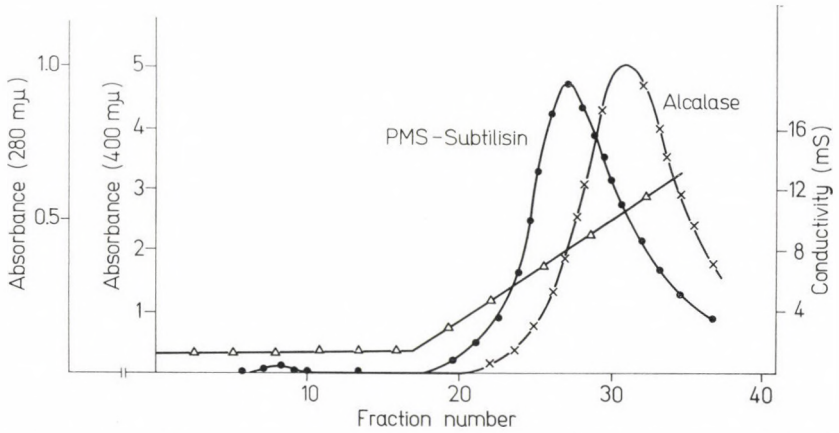

Fig. 1. Chromatography of 90 per cent PMS-subtilisin and 10 per cent alcalase at pH 6.5 using linear gradient elution with phosphate buffers. ● = protein concentration (E_{280}); x = enzymatic activity ($\triangle E_{420}$); △ = conductivity (mS)

the total absorption at 280 mμ of the commercial preparation could be recovered as pure alcalase.

In contrast to subtilisin (Polgár, Bender, 1969), no autodigestion of alcalase was noticeable during the chromatographic procedure. Namely, re-chromatography of the pure alcalase fraction after lyophilization and gel filtration resulted in a single protein peak and practically no degradation products could be detected in the column eluate.

The chromatographic behaviour of alcalase is similar to that of subtilisin, but alcalase binds stronger to CMC than subtilisin. The chromatographic pattern of a 9 : 1 mixture of PMS-subtilisin and alcalase is illustrated in Fig. 1. The inactive derivative of subtilisin is chromatographically identical with subtilisin:

these two proteins are not separated under the conditions of the chromatography (Polgár, Bender, 1969). PMS-subtilisin, which is present in great excess, accounts for the first peak (measured at 280 mμ) while the second peak containing the enzymatic activity towards NZG is alcalase (measured by its enzymatic activity).

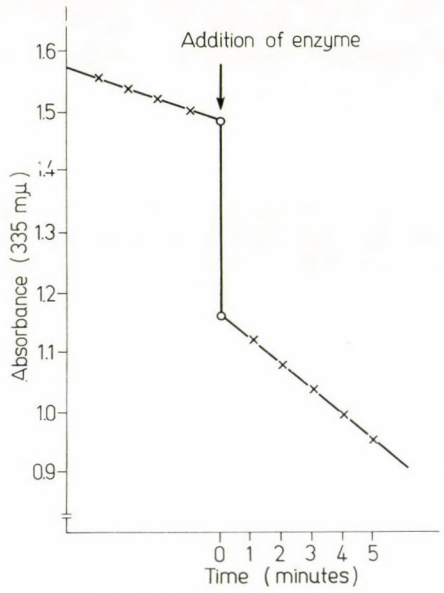

Fig. 2. Titration of alcalase with *N-trans*-cinnamoylimidazole

Titration of the active site of alcalase. In order to determine the concentration of alcalase we have chosen the active site titration method as already used with different hydrolytic enzymes (Bender et al., 1966). This method yields the actual active enzyme concentration the knowledge of which is indispensable for the calculation of true kinetic constants.

The alcalase-catalyzed hydrolysis of *N-trans*-cinnamoylimidazole exhibits an initial "burst" in the decrease of absorption of *N-trans*-cinnamoylimidazole followed by a steady-state (zero order) decrease of substrate concentration This observation provides convincing evidence for a stepwise mechanism of alcalase action, involving an acylenzyme intermediate. Accordingly, the reaction sequence is given by Eq. 1:

$$E + S \overset{K_S}{\rightleftharpoons} ES \xrightarrow{k_2} E'S \xrightarrow{k_3} E + P_2 \qquad (1)$$
$$\Big\downarrow +$$
$$P_1$$

where the symbols have their usual meaning (Bender, Kézdy, 1965).

The concentration of the active site in a solution of alcalase has been determined from the initial (negative) "burst" shown in Fig. 2. In distinction from the

titration of subtilisin (Bender et al., 1966), alcalase is titrated at pH 5.0 instead of pH 7.0 because the rate of de-cinnamoylation of alcalase is considerably higher than that of subtilisin and so an accurate extrapolation at zero time cannot be achieved at the higher pH value. Though at pH 5.0 the rate of spontaneous hydrolysis of *N-trans*-cinnamoylimidazole is somewhat higher and the molar extinction coefficient of the titrant varies somewhat with pH, the results of the titration are reproducible within an error of $\pm$ 5 per cent, provided that the concentration of the titrated enzyme is sufficiently high. Under the conditions of the titration, substrate concentration is much higher than K_m which means that the hydrolysis takes place at saturation. Furthermore, the "burst" is completed in a few seconds which indicates that the rate constant of acylation (k_2) is much higher than the rate constant of deacylation (k_3).

Preparation of thiol-alcalase. In principle, thiol-alcalase was prepared similarly to thiol-substilisin (Polgár, Bender, 1966; Neet, Koshland, 1966). As the first step, the seryl residue at the active site was reacted with PMS fluoride, and then the PMS group was displaced by thiolacetate ion. The acetyl-thiol-alcalase formed in this way was finally hydrolyzed by the very catalytic entity of the enzyme

Alcalase reacted rapidly and stoichiometrically with PMS fluoride present in a slight excess at pH 7.0–7.5. PMS-alcalase formation was completed within half an hour as indicated by 99.9 per cent inhibition of NZG hydrolysis.

PMS-1'-^{14}C fluoride was used in studies of the displacement of the PMS group from PMS-alcalase by thiolacetate ion. Samples of the reaction mixture were precipitated with acetone, and the radioactivity bound to the protein was measured after washing. In contrast to subtilisin, the release of the PMS group from PMS-1'-^{14}C-alcalase could not be significantly increased by conducting the reaction at slightly acidic pH. While there was an about 4.5 fold rate enhancement with subtilisin when the pH was decreased from 6.5 to 5.3, the corresponding increase in rate was only 1.4 fold with alcalase.

Because of the slow reaction of PMS-alcalase with thiolacetate ion, preparation of thiol-alcalase at room temperature did not prove to be convenient ($t_{1/2}$

Table 2

*Rate constants for displacement of PMS-group from PMS-alcalase by thiolacetate ion at different temperatures**

t, °C	k, hour^{-1}	$t_{1/2}$, hour
24	0.0145	47.5
30	0.031	22.2
37	0.093	7.4
45	0.256	2.7

* in 0.8 M thiolacetate solution at pH 6.0

was about 50 hours in 0.8 M thiolacetate at pH 6.5). Therefore, we attempted to utilize the greater heat stability of alcalase. Fig. 3 shows the release of the PMS group from PMS-1'-^{14}C-alcalase at pH 6.0 at different temperatures. It can be seen that the reactions follow first order kinetics. The rate constants are compiled in Table 2.

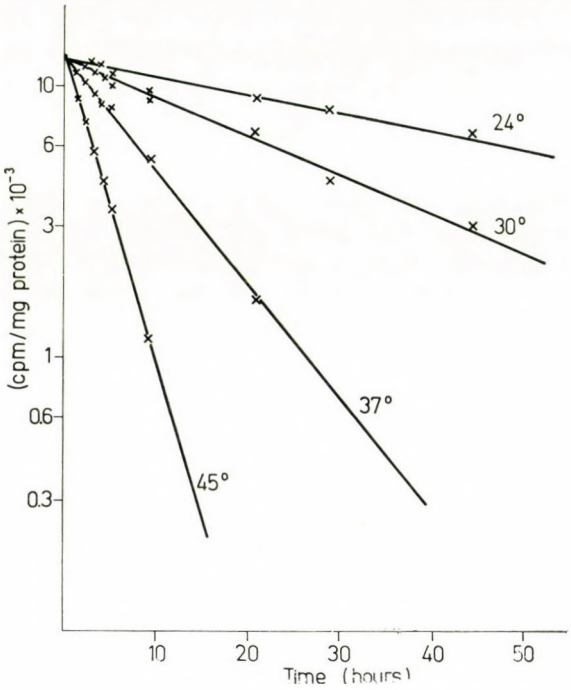

Fig. 3. Reaction of PMS-1'-^{14}C-alcalase with thiolacetate ion at pH 6.0 at different temperatures

Fig. 3 and Table 2 show a considerable rate enhancement with increasing temperature. This is about 18-fold when the temperature is elevated from 24 °C to 45 °C. Accordingly, the preparation of thiol-alcalase at elevated temperature is obviously the method of choice, comparable to the lowering of pH used for the preparation of thiol-subtilisin. An overnight reaction at 45 °C is already sufficient for a good conversion of alcalase into its thiol derivative, while a 2-day incubation at pH 5.5 has been employed with subtilisin.

A plot of log k *vs.* 1/T in Fig. 4 shows that the displacement of PMS group by thiolacetate ion obeys Arrhenius' law. One can calculate from the figure that Q_{10} is about 3.8, i.e. an increase of 10 °C in temperature enhances the rate constant 3.8 times.

Cinnamoyl-thiol-alcalase formation. Evidence of the transformation of serine into cysteine side chain by the above method is obtained by studying the forma-

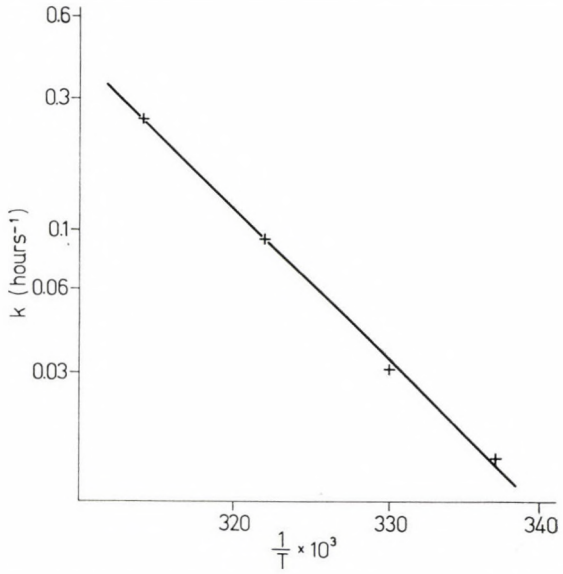

Fig. 4. Arrhenius plot of the rate constant of the reactions of PMS-alcalase with thiolacetate ion

tion of acyl-derivatives using *N-trans*-cinnamoylimidazole. It is known that cinnamoyl-O-esters exhibit an absorption maximum at a significantly lower wavelength than cinnamoyl-S-esters (Bender, Kézdy, 1965). The difference spectrum of cinnamoyl-alcalase *vs.* alcalase and that of cinnamoyl-thiol-alcalase *vs.* thiol-alcalase is shown in Fig. 5. As expected, the difference spectrum of cinnamoyl-alcalase has a maximum around 288 mμ, while the corresponding maximum for the thiol-derivative is observed at 307 – 308 mμ. This is similar to our previous findings with subtilisin and thiol-subtilisin, respectively (Polgár, Bender, 1966). The spectra in Fig. 5 were measured at pH 4.0, because cinnamoyl-alcalase rapidly hydrolyzes at higher pH values. Even at pH 4.0 an extrapolation to zero time was necessary with alcalase.

Chromatography of thiol-alcalase. In order to isolate thiol-alcalase free of contamination by alcalase, which would interfere with kinetic investigations, ion exchange chromatography was employed. As described above, alcalase binds to CMC equilibrated with 0.01 M phosphate buffer, pH 6.5, and it can be eluted by increasing the concentration of the buffer. In contrast to alcalase, thiol-alcalase passes through the CMC column equilibrated with 0.01 M phosphate buffer, pH 6.5. Despite a marked tailing of the thiol-alcalase fraction, the chromatographic method is suitable for the alcalase-free preparation of the thiol-enzyme.

Hydrolysis of NPA catalyzed by alcalase and thiol-alcalase. The enzymatic properties of alcalase and thiol-alcalase were studied by using NPA as substrate. The kinetics of the alcalase-catalyzed hydrolysis of NPA is similar to

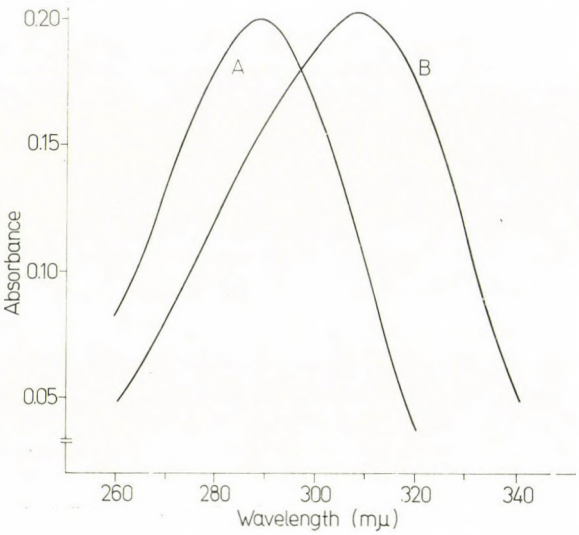

Fig. 5. The difference spectra of *N-trans*-cinnamoyl-alcalase *vs.* alcalase (curve A) and *N-trans*-cinnamoyl-thiol-alcalase *vs.* thiol-alcalase (curve B) at pH 4.0 0.1 M acetate buffer. *N-trans*-cinnamoylimidazole (9.55×10^{-6} M) was treated with excess enzyme (2×10^{-5} M), to transform completely the *N-trans*-cinnamoyl-imidazole to the cinnamoyl-enzyme

those of chymotrypsin, subtilisin and papain, and presumably takes place according to Eq. 1, as described above in connection with the active site titration. The rate of the steady-state portion of the hydrolysis is a hyperbolic function of substrate concentration. The kinetic parameters k_{cat} and K_m were

Table 3

*Kinetic constants of the hydrolysis of NPA catalyzed by alcalase**

pH	Buffer (0.1 M)	k_{cat} (sec^{-1})	K_m (10^4 M)	k_{cat}/K_m (M^{-1} sec^{-1})
5.95	phosphate	0.121	8.77	138
6.52	phosphate	0.294	7.95	370
7.00	phosphate	0.63	8.15	775
7.43	phosphate	0.75	5.50	1360
7.64	veronal	1.02	5.22	1950
7.80	phosphate	0.96	5.32	1810
8.48	veronal	1.11	5.75	1910
8.87	veronal	1.02	4.72	2160

* $25.5 \pm 0.2°$; 3.3 per cent (v/v) acetonitrile-water; alcalase 9.0×10^{-6} M; NPA 18.8×10^{-4} M.

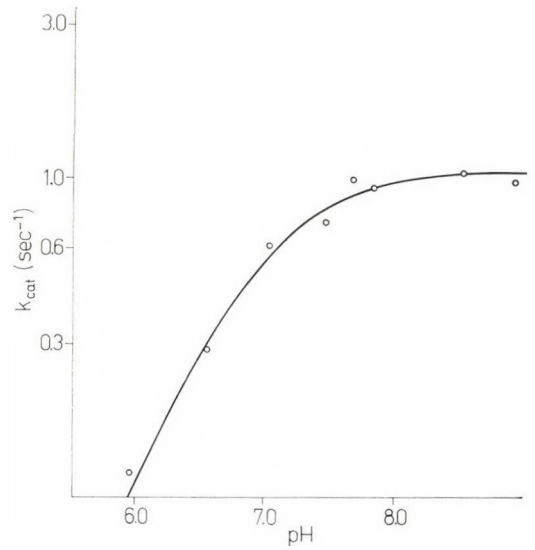

Fig. 6. pH dependence of the k_{cat} of NPA-hydrolysis catalyzed by alcalase

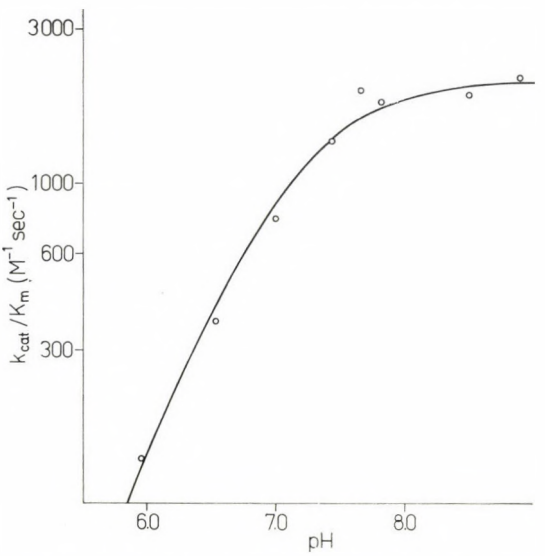

Fig. 7. pH dependence of the k_{cat}/K_m of NPA-hydrolysis catalyzed by alcalase

calculated from Lineweaver-Burk plots, and a second-order rate constant (Kézdy, Bender, 1962) was calculated by dividing k_{cat} by K_m. Table 3 and Figs 6 and 7 show the pH dependence of k_{cat} and k_{cat}/K_m of the alcalase-catalyzed reaction. Assuming that these pH-dependencies are due to one ionizable group, k(limit)

and pK values can be calculated from the curves. These data are compiled in Table 4.

Exact kinetic constants of the thiol-enzyme reactions were not determined. Unfortunately, the hydrolysis of NPA catalyzed by chromatographically purified

Table 4

Kinetic constants of the hydrolysis of NPA catalyzed by alcalase

	k (limit)	pK
k_{cat}	1.1 ± 0.1 sec^{-1}	6.95 ± 0.15
k_{cat}/K_m	2100 ± 200 M^{-1} sec^{-1}	7.15 ± 0.15

thiol-alcalase was only inhibited 60–70 per cent by PMB. This phenomenon is similar to that found for the thiol-subtilisin-catalyzed hydrolysis of NZG (Polgár, Bender, 1966, 1969).

Despite the additional hydrolytic activity of thiol-alcalase, it can be estimated that k_{cat} for the hydrolysis of NPA catalyzed by the thiol-derivative is at least 30 times lower, than the corresponding value for the reaction catalyzed by the native enzyme.

Discussion

From the point of view of chemical behaviour, the sulfhydryl group in thiol-alcalase may be equally, or even more, reactive than the hydroxyl group. Furthermore, it cannot be expected that the subtitution of one atom in a large protein molecule with a similar one would cause an easily measurable alteration in some of its physical parameters. Nevertheless, studies on thiol-subtilisin revealed that replacement of an oxygen atom at the active site of a "serine enzyme" with a sulfur atom resulted in a pronounced change both in the enzymatic activity and in the chromatographic behaviour of the protein (Polgár, Bender 1966; Neet, Koshland, 1966; Polgár, Bender, 1967; Polgár, Bender, 1969). However, it could not be decided whether a unique phenomenon was observed with thiol-subtilisin, or the active site was a highly sensitive region of most enzymes, the modification of which could affect not only the catalytic process itself, but also some of the physical properties of the protein molecule. Therefore, we chose alcalase, both the stability and the reactivity of which were known to be different from those of subtilisin. Thiol-alcalase was prepared from alcalase and the properties of thiol-subtilisin and thiol-alcalase were compared.

The rate of reaction of PMS-subtilisin with thiolacetate ion is greatly increased in slightly acidic medium. On the other hand, protonation of PMS-alcalase does not significantly promote the reaction with thiol-acetate. This differ-

ence may reflect the fact that the active site of alcalase is much less affected by protonation than the active site of subtilisin.

Due to the high stability of alcalase against heat inactivation, the reaction of PMS-alcalase with thiol-acetate ion can be carried out at elevated temperature. The temperature dependence of this reaction conforms to Arrhenius' equation. This indicates the absence of a gross change in the reaction mechanism between 24 °C and 45 °C.

Alcalase catalyzes the hydrolysis of NPA with a higher rate than subtilisin. The value of k_{cat} of the alcalase-catalyzed reaction is about 3.5 times higher. The second-order rate constant of the same reaction, k_{cat}/K_m is 3.1 times higher than the corresponding value for subtilisin. This means that within experimental error there is the same difference between the two rate constants when subtilisin and alcalase are compared. This conclusion is supported by the finding that there is no difference in the K_m for the two enzymes (5×10^{-4}M). Since k_{cat}/K_m is equal to k_2/K_S (Bender, Kézdy, 1965), either the acylation constant is increased, or the equilibrium constant of the enzyme-substrate complex is decreased with alcalase, as compared to the subtilisin-catalyzed reaction.

Despite the difference in stability and in kinetic properties between subtilisin and alcalase, the transformation of the two enzymes into the corresponding thiol derivative results in similar changes. The binding capacity of the two thiol-enzymes to CMC decreases essentially to the same extent. Likewise, the overall rate constant of NPA hydrolysis is similarly decreased, and also an additional hydrolytic activity (Polgár, Bender, 1969) is encountered in both thiol-enzymes. As in the case of subtilisin, NZG is a much better substrate of alcalase than NPA. The catalytic activity of thiol-alcalase towards NZG is very low similarly to thiol-subtilisin and it cannot even be accurately measured because of the high inherent hydrolytic activity of thiol-alcalase which is not inhibited by PMB.

Since the results described in this paper conform with those found with subtilisin, the observed changes in the catalytic properties of subtilisin, which are due to a subtle modification in its active site, do not reflect a special case. Possibly, it is a general rule that the maximal enzymatic reaction rate which is obtained with a highly specific substrate is so much dependent on the steric fit between substrate and the catalytic groups of the enzyme, that even a minute distortion which could result from the difference between the atomic radii of oxygen and sulfur ($\sim$ 0.4 Å) will lead to a marked decrease of the overall rate.

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Temperature and Concentration Dependence of the Stability of Pig Lactate Dehydrogenase Isoenzymes H_4, H_2M_2 and M_4

Non-Intermediate Heat Stability of the Hybrid Tetramer

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The rate of inactivation of three isoenzymes of pig lactate dehydrogenase is compared at elevated temperature, in 0.2 M tris/HCl buffer, pH 7.5. As a consequence of varying temperature in the range 44—62(64)°C, half-lifes ranging from about 1 week to about 1 minute are obtained.

The rate of inactivation of isoenzyme H_4 is not affected by protein concentration in the range 0.01 to 1 mg/ml. With isoenzyme M_4, a protective mechanism appears to be operating at higher protein concentrations, the effect of which becomes less marked as temperature is increased. With the hybrid tetramer, a more complex picture is obtained. In the range 44—52 °C the rate constant is lowered by increasing protein concentration, while at higher temperatures the situation is reversed.

Temperature dependence of the rate of inactivation of the three isoenzymes is distinctly different. For this reason, it depends on temperature whether isoenzyme H_4 is less stable than isoenzyme M_4 (lower temperature range), or more so (higher temperature range). However, in the whole range of temperature, and within the whole range of studied protein concentrations, isoenzyme H_2M_2 is less stable than both homotetramers H_4 and M_4.

One of the widely used procedures by which molecular species of proteins in solution are characterized is the determination under well-defined conditions of their heat stability. It appears, that the heat stability defined as the reciprocal of the first-order rate constant of inactivation is a sensitive index of protein conformation, since it is greatly affected by pH, ionic composition and ionic strength of the solution, the binding to the protein of specific ligands (substrates, allosteric effectors), and so on (see, e.g. Freundlich, Umbarger, 1963). It is further clear, that even small variations in the amino acid sequence, such as a single substitution due to point mutation, can lead to a dramatic change in heat stability (e.g. Schlesinger et al., 1963). These observations would lead to the conclusion that the diverse types of interactions which contribute to the maintenance of the specific metastable state of the steric structure of a protein molecule might be all reflected in its stability. Accordingly, one would expect that the stability of a protein which is composed of a number of polypeptide chains is likely to be affected by inter-chain interactions. Indeed, it would also follow that the heat inactivation of such oligomeric proteins might be a considerably complex reaction.

To test these ideas we have studied the heat inactivation of three isoenzymes of pig lactate dehydrogenase. Following the initial observation by Appella and Markert (1961) it is, by now, well established that the commonly observed isoenzymes of vertebrate lactate dehydrogenases are tetramers composed of four polypeptide chains. It is further known (Markert, Appella, 1963) that the five isoenzymes represent the five possible combinations of two different polypeptide chains, to which we shall refer (after Fondy et al., 1964) as subunit H, and M, respectively. In choosing lactate dehydrogenase as the model protein we have been led by the obvious advantage offered by the existence of five electrophoretically separable isoenzymes, and also by the fact that in aqueous solution the aggregational state of this protein appears to be a very stable tetramer.

The results presented in this paper tend to support the ideas outlined above. It will be shown that, as a general rule, the rate of inactivation does depend on protein concentration. It will be further demonstrated that the stability of the heterotetramer H_2M_2 is not intermediate between those of the homotetramers H_4 and M_4. This latter observation indicates that interactions among the four polypeptide chains (subunits) of lactate dehydrogenase do affect the stability of the protein, in apparent contradiction with the conclusion of a number of publications (Plagemann et al., 1961., rabbit enzymes; Wachsmuth, Pfleiderer, 1963, human lactate dehydrogenases; Fondy et al., 1964, chicken and beef isoenzymes). We show in two further communications of this series (Südi, 1968, 1969) that at least part of the inter-subunit interactions observed with pig lactate dehydrogenase isoenzymes can be easily accounted for by considering the role which the tetrameric structure of the protein is playing in the mechanism of inactivation.

Materials and Methods

Enzymes. Pig muscle lactate dehydrogenase (isoenzyme M_4) was prepared as described by Jécsai (1961). The pig heart enzyme (isoenzyme H_4) was obtained by a modification of Straub's procedure (Straub, 1940). In the modified procedure 0.75 part (v/v) of cold acetone (-15 °C) is added to the ice-water cooled solution of the enzyme in 0.1 M phosphate buffer and left then to stand for $1-2$ hours in an ice-water bath. The final preparations of the two homotetramer enzymes were three, and two times recrystallized, respectively, and both contained a negligible contamination with other isoenzymes, as judged from the results of analytical chromatography on DEAE cellulose.

Isoenzyme H_2M_2 was prepared from the crystalline homotetramers H_4 and M_4 by the freeze-thaw method as described by Chilson et al. (1964). The hybridization mixture was thoroughly dialyzed against 0.05 M Tris/HCl buffer, pH 7.5, and this solution was charged onto a column of DEAE cellulose (Whatman). Elution was done with a linear or convex gradient of sodium chloride in the same buffer. A typical elution pattern is shown in Fig. 1. The chromatographic peaks were identified by a comparison of their electrophoretic mobility with those found

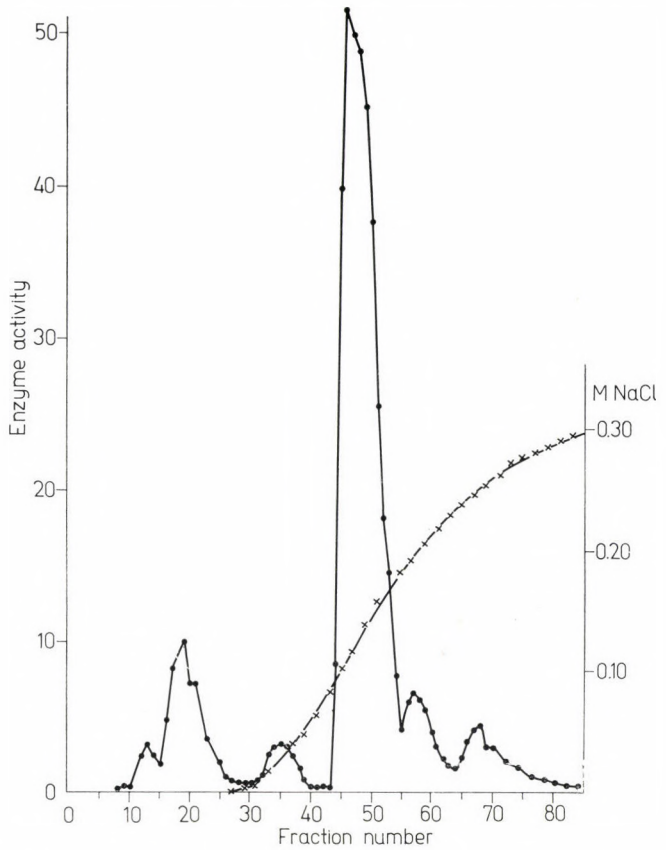

Fig. 1. Isolation of the hybrid H_2M_2 by chromatography on DEAE cellulose. Load: hybridization mixture prepared of 400 mg of each of isoenzyme H_4 and M_4. Enzyme activity (●—●) is determined with 1 mM pyruvate in the reaction mixture and expressed in arbitrary units. Concentration of NaCl (x—x) is determined by conductometry. Volume of the column is 240 ml; it is equilibrated with 0.05 M tris/HCl buffer, pH 7.5, and eluted with the indicated gradient of NaCl in the same buffer. The five main activity peaks of the elution profile are, as identified by disc electrophoresis on polyacrylamide gel, isoenzyme M_4 (not bound), M_3H (0.01 M NaCl), H_2M_2 (0.09 M NaCl), H_3M (0.18 M NaCl), and H_4 (0.24 M NaCl), respectively

in a hybridization mixture containing all five isoenzymes. It can be seen from Fig. 1 that the chromatographic procedure is suitable for the resolution of isoenzyme H_2M_2. It is also seen from Fig. 1 that the symmetrical hybrid H_2M_2 is formed preferentially over those of isoenzymes HM_3 and H_3M. In our experience, this is a reproducible result obtained when pig lactate dehydrogenase isoenzymes are hybridized by the freeze-thaw method. The fractions which contained isoenzyme H_2M_2 were pooled, and the enzyme was precipitated by the addition of 30 g solid

ammonium sulphate per 100 ml of the solution. The ammonium sulphate suspension was stable for months on storage at 4°C, stability including the absence of any change in the isoenzyme pattern of the preparation.

Analytical chromatography. When a determination of the distribution of total enzyme activity among the isoenzymes present in a mixture was needed, this was done by chromatography on DEAE cellulose. Analytical chromatography was carried out similarly to the preparative procedure described above, routinely with a total amount of about 1 mg protein.

Gel electrophoresis. Identification of the isoenzymes was achieved by disc electrophoresis on polyacrylamide gel as described by Davis (1964). Staining for protein was done with the dye Amidoblack, and staining for lactate dehydrogenase activity with a solution of NAD, lactate, phenazine methosulfate and the dye nitrobluetetrazolium (Markert, Møller, 1959).

Protein content and enzyme activity. Optical density in 0.1 N NaOH at 280 mμ was routinely used for the determination of protein concentration. The optical density of 1 mg/ml solutions of all three isoenzymes was found to be 1.29, with 1-cm optical path and under the above conditions in agreement with Jécsai (1961). Molecular weight of the tetramers was assumed to be 140 000 (Jenicke, Knof, 1968).

Enzyme activity was routinely determined at room temperature, with 0.01 – 0.2 μg/ml of enzyme protein, 0.133 mM NADH and 1 mM pyruvate in the assay mixture, which was buffered to pH 7.5 with 0.2 M Tris/HCl buffer. At 25 °C, the molecular activity of the three isoenzymes was found to be 15 600, 27 700, and 44 100 moles of NADH per minute per mole of the tetramer H_4, H_2M_2, and M_4, respectively. It was further found that, by varying the concentration of pyruvate under these standard conditions, the ratio of activities determined with 1 and 10 mM pyruvate in the assay mixture was 2.7 for isoenzyme H_4, 1.59 for isoenzyme H_2M_2 and 1.08 for isoenzyme M_4. Even though these values showed some inter-experimental variation in routine experiments carried out at varying room temperature, they were useful as an additional means for characterizing the three isoenzymes (for references see Rouslin, Braswell, 1968).

Heat treatment. The solutions in which enzyme was exposed to elevated temperature in these studies contained 0.2 M tris/HCl buffer, pH 7.5. Stock solutions of the enzymes from which the required dilutions were made were obtained by centrifugation of the crystal suspension, and gel-filtration (Sephadex G-75, Pharmacia) in tris/HCl buffer of the dissolved precipitate. Depending on the rate of inactivation, samples had to be withdrawn from the constant-temperature water bath at time intervals ranging from 30 seconds to 24 hours. In order to obtain well-defined heat treatments the following procedure was found to be convenient: 1-ml aliquots of the solution were pipetted into uniform test tubes held by a rack kept in an ice-water bath. Treatment was initiated by immerging the rack into a well-circulated constant temperature bath. Following equilibration, the tubes were sealed with parafilm in case of treatments lasting longer than about 10 minutes. At the required time intervals one of the tubes was withdrawn from the bath and

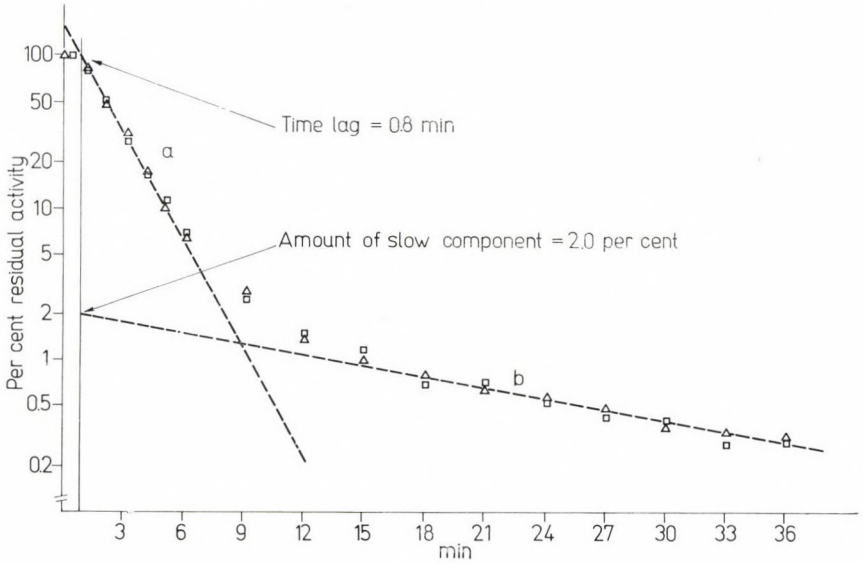

Fig. 2. Prolonged time course of the heat inactivation of isoenzyme M_4 at 60 °C, illustrating the way in which correction for the experimental time lag is made, and the way in which the amount of a slower inactivated minor component has been estimated. Results obtained with initial protein concentrations of 17 mg/ml (△) and 1 mg/ml (□). Line *a* shows the progress of inactivation of the bulk of the enzyme. The experimental time lag is defined as the intercept of this straight line with the 100 per cent-activity ordinate. A second straight line (line *b*) is drawn to the points obtained between 18 and 36 minutes. A minor component of the sample is assumed to be responsible for this slow rate of inactivation, the amount of which is estimated by extrapolating line *b* to 0 min., with correction for the time lag

rapidly immerged into an ice-water bath. It is obvious that by following this procedure, two intervals of temperature-transition have to be corrected for, and that these are the same for all samples, irrespective of the duration of the heat treatment proper; heating-up time, and cooling-down time, respectively. Indeed, it was found in relatively rapid reactions that the joint correction under these conditions was about minus 0.7 – 1.2 minutes (see Fig. 2).

The progress of inactivation was followed by determining residual enzyme activity in the successive samples. Residual activity was expressed as per cent of the initial activity, the logarithm of these values was plotted against time, the best-fitting straight line was drawn by free hand to usually 5 – 8 experimental points, and from the straight line of the semilogarithmic plot a first-order rate constant was calculated.

Results and Discussion

It has been the purpose of these studies to establish the temperature and concentration dependence of the stability of isoenzymes H_4, H_2M_2 and M_4. In the experiments which follow this has been done in the temperature range 44 – 64 °C,

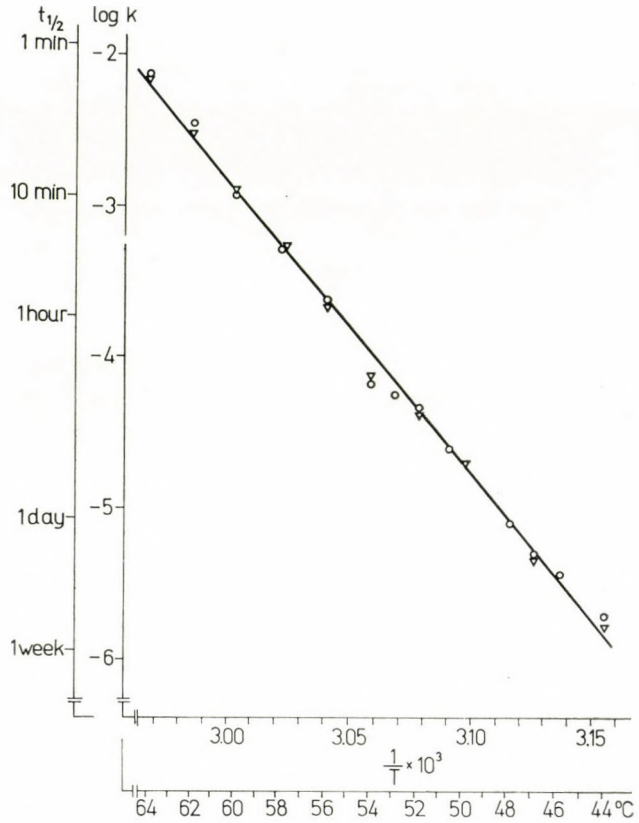

Fig. 3. *Arrhenius plot* of the first-order rate constant of inactivation of isoenzymes H_4 (a) and M_4 (b). Initial protein concentration is 0 01 mg/ml (△) and 1 mg/ml (o), respectively. A second ordinate gives the corresponding half-life of the enzyme. A second abscissa is also drawn to show temperature in Celsius. In Fig. 3b the *Arrhenius function* obtained for isoenzyme H_4 is shown by the broken line for the sake of a convenient comparison

with initial protein concentrations ranging from 0.01 to 1 mg/ml. The technical details of these experiments are given above, to these some further remarks should be added, which are relevant to the main conclusions of this paper.

(1) Stability is defined as the reciprocal of the rate constant of the loss of enzyme activity. It was observed that when initial protein concentration was higher than about 0.1 mg/ml, a visible precipitate was formed parallel with the loss of enzyme activity. In routine experiments these suspensions were diluted for the activity assay without centrifugation. It was found in control experiments that separation of the precipitate by sedimentation did not affect the activity of the solution. On the other hand, it was further established that some of the inactivated protein did not precipitate, since the specific activity (O. D. 340 mμ per time per

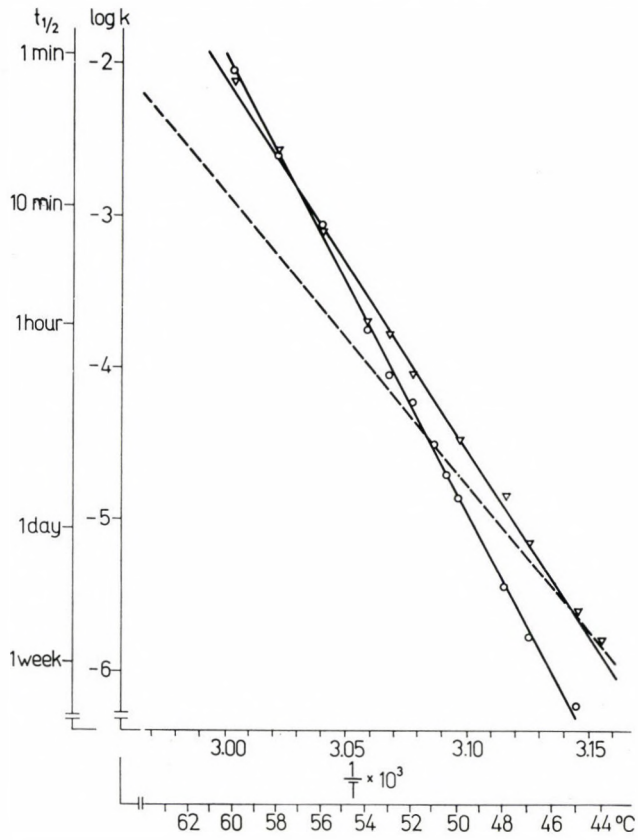

Fig. 3b

O. D. 280 mμ) of the clear supernatant of partially inactivated samples was consistently lower than that of the original enzyme.

(2) It should be stressed that apparent conformation of the data with the first-order rate equation was only observed down to about 10 – 5 per cent residual activity. The residual activity left after this initial phase was always lost somewhat slower. It could be shown in special experiments carried out with isoenzyme M_4 at 60 °C (Fig. 2) that the slowly inactivated component is responsible for about 2 per cent of the total activity with a wide range of initial protein concentrations. It was not further investigated whether this more stable minor component was present as an inhomogeneity in the original enzyme preparation, or only obtained in the course of the initial phase of inactivation as a by-product. In similar experiments with isoenzyme H_4 an analogous minor component was found which comprised about 1.5 per cent of the total activity. In most experiments with the hybrid tetramer a similar picture was also obtained for isoenzyme H_2M_2. However,

under certain conditions the apparently first-order initial phase of inactivation appeared to be somewhat shorter, extending not farther than about 60–50 per cent residual activity. The possible nature of this apparent inhomogeneity is shown in another communication (Südi, 1968). The rate constants given in this paper always refer to the initial phase of inactivation.

With these specifications, and under the described conditions the heat stability of isoenzymes H_4, H_2M_2 and M_4 was found to be as follows.

Stability of isoenzyme H_4. Initial protein concentration does not apparently affect the rate of inactivation of isoenzyme H_4 in the whole range of temperature studied. This is shown in Fig. 3a, in which the results obtained with 0.01 and 1 mg/ml initial protein concentration are shown as the *Arrhenius plot* of the first order rate constant of inactivation. It should be noted that this conclusion is valid under conditions when the rate constant varies within five orders of magnitude.

It further appears from Fig. 3a that the experimental points fall on a straight line in the *Arrhenius plot*. This indicates that there may be no gross change in the mechanism of inactivation of isoenzyme H_4 between 44 and 64 °C. The mechanism of inactivation may be characterized by an *Arrhenius activation energy* of 89 000 calories per mole.

Stability of isoenzyme M_4. As shown in Fig. 3b, a more complex picture is obtained for isoenzyme M_4. It is seen that there is a marked difference in the rate constant of inactivation at high and low protein concentration (1 mg/ml, and 0.01 mg/ml, resp.), when temperature is lower than about 53 °C. A further characterization of the concentration dependence of the rate of inactivation of isoenzyme M_4 is given in Table 1. It is seen from the table that at 50 °C the half-life of the

Table 1

Effect of protein concentration on the half-life of isoenzyme M_4

Initial protein concn.	Half life	
	50 °C	58 °C
0.01 mg/ml	330 min	4.7 min
0.1 mg/ml	330 min	5.2 min
1 mg/ml	730 min	4.1 min
5 mg/ml	1100 min	—
10 mg/ml	—	3.8 min

enzyme is lower at low protein concentrations, and that a concentration effect is only apparent at protein concentrations higher than 0.1 mg/ml. Table 1 also shows that no clear concentration dependence can be ascertained at 58 °C.

It can be calculated from the straight lines shown in Fig. 3b that the *Arrhenius activation* energy of the inactivation of isoenzyme M_4 is 112 000 calories

per mole in a 0.01 mg/ml solution, and 137 000 calories per mole in a 1 mg/ml solution. These values are both significantly higher than the calculated activation energy or the inactivation of isoenzyme H_4. This difference in the activation energies has a further consequence. It is seen from Fig. 3b that the *Arrhenius functions* for the two homotetramer isoenzymes intersect, at about 51, and 45 °C, respectively, depending on protein concentration. Fig. 3b shows that especially marked difference is obtained at temperatures above 55 °C, irrespective of protein concentration, and also at temperatures lower than about 48 °C, but only at a high protein concentration. It is interesting to note that the relative stability of the two isoenzymes is inversed in these two temperature ranges, and that in the low-temperature range, where the half-life of the two enzymes varies somewhere between 1 day and 1 week, isoenzyme M_4 is markedly more stable than isoenzyme H_4. This finding is at variance with an apparently general conclusion in the literature (Kaplan, 1965), according to which the heart-type isoenzyme of the lactate dehydrogenase of most vertebrate species is more stable at elevated temperature than the muscle-type isoenzyme.

Stability of isoenzyme H_2M_2. Results obtained in similar experiments with the hybrid tetramer H_2M_2 are shown in the *Arrhenius plot* of Fig. 4. For the sake of convenient comparison, results obtained with isoenzyme H_4 and M_4 are also shown in the figure. It is apparent at first sight that in the whole range of temperature the stability of the hybrid tetramer is lower than the stability of both corresponding homotetramers.

It is seen from Fig. 4 that at low protein concentration an apparently straight-line *Arrhenius function* is obtained, from which an activation energy of 80 000 calories per mole can be calculated. At high protein concentration the situation appears to be more complex, the corresponding *Arrhenius function* being a curve with an inflexion point at about 54 °C, which intersects the straight line obtained for low protein concentration at about 53 °C. It is also illustrated by the more detailed findings in Table 2 that, at high temperatures where the half-life of the enzyme is in the 1 – 10 minutes range, higher concentrations are increasingly less stable, while in the temperature range where the half-life varies from a few hours to a few days (temperatures lower than 53 °C), stability is markedly increased at protein concentrations higher than about 0.2 mg/ml. It should be noted that in the lower temperature range the concentration dependence of the rate of inactivation of isoenzymes H_2M_2 and M_4 appears to be similar (Tables 1 and 2).

We suggest that the non-intermediate stability of the hybrid tetramer shown in Fig. 4 deserves special attention, since this finding indicates that interactions among the subunits of the protein affect its stability. Speaking in terms of interactions, one may conclude that stability of the hybrid tetramer is somehow impaired by unfavourable interactions among the two types of subunits. The possible implications of this finding are obvious, indeed, in two further papers of this series (Südi, 1968, 1969) we have used it as a starting point for investigations on the possible role of the tetrameric structure in the mechanism of inactivation.

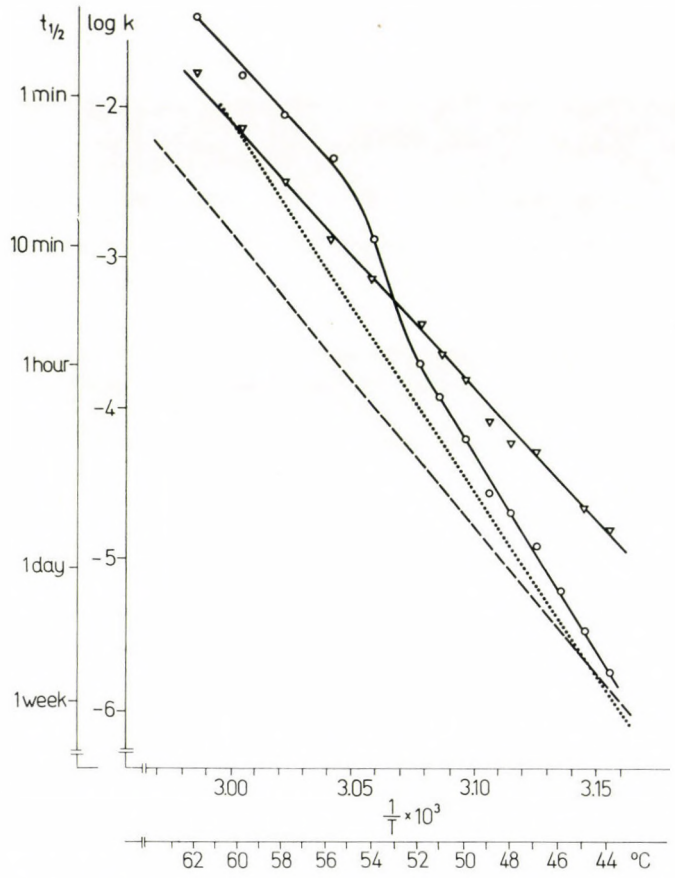

Fig. 4. *Arrhenius plot* of the first-order rate constant of the initial inactivation of isoenzyme H_2M_2. Initial protein concentration is 0.01 mg/ml (▽), and 1 mg/ml (o), respectively. The *Arrhenius functions* obtained for isoenzyme H_4 (Fig. 3a) and for 0.01 mg/ml of isoenzyme M_4 (Fig. 3b) are shown by the broken line, and the dotted line, respectively. Plot drawn as Fig. 3

Table 2

Effect of protein concentration on the half-life of isoenzyme H_2M_2

Initial protein concn.	Half life	
	51 °C	58 °C
0.01 mg/ml	45 min	3.5 min
0.1 mg/ml	—	2.8 min
0.2 mg/ml	45 min	—
1 mg/ml	100 min	1.3 min
8.6 mg/ml	360 min	—

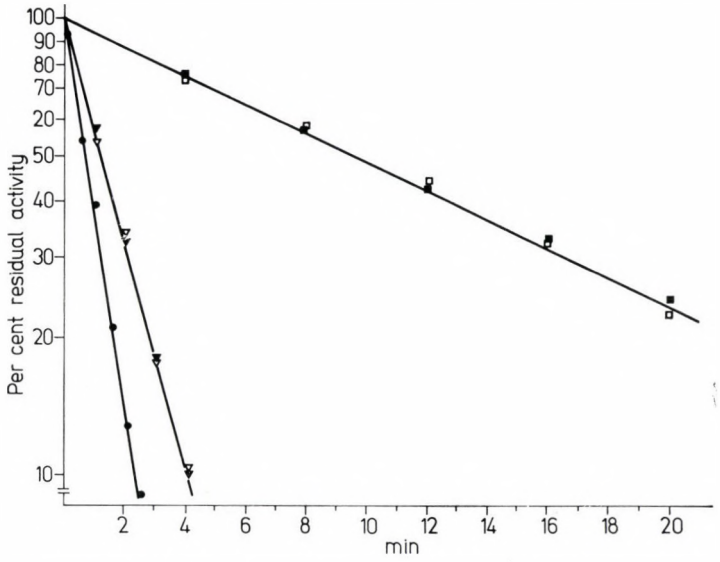

Fig. 5. Stability of 1 mg/ml solutions of isoenzymes H_4 (□, ■), M_4 (▽, ▼) and H_2M_2 (●) at 60 °C. Open symbols denote homotetramer enzymes not subjected to the freeze-thaw procedure. Filled symbols stand for isoenzymes isolated by chromatography from a hybridization mixture similar to that shown in Fig. 1

It is shown in the same papers that the nature of the operating mechanism can be characterized by studying the stability of the isoenzyme pattern in the course of inactivation.

Here we should like to deal with one further relevant question. Namely, it may be asked whether the observed lower stability of the hybrid tetramer H_2M_2 is also characteristic of isoenzyme H_2M_2 directly isolated from pig tissues, or only obtained as an artefact in these studies because we have applied an *in vitro* hybridization procedure for the preparation of the enzyme. At present, we only have data on the stability of isoenzyme H_2M_2 obtained by *in vitro* hybridization. However, it is generally accepted that homotetramers present in hybridization mixture represent tetramers which have been reversibly dissociated under the conditions which have led to hybridization (Appella, Markert, 1961; Chilson et al., 1964; Anderson, Weber, 1966). Therefore, we have re-isolated isoenzymes H_4 and M_4 from a hybridization mixture used for the preparation of isoenzyme H_2M_2, and subsequently compared their heat stability to that of the original enzymes. Results obtained at 60 °C are shown in Fig. 5. It is seen that the stability of neither homotetramer is affected by the freeze-thaw and chromatographic procedures. This observation strongly supports the view that the observed low stability of isoenzyme H_2M_2 is not likely to be due to dissociation and re-association of its subunits in the course of *in vitro* hybridization.

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Amino Acid Sequence of Fibrin Glycopeptides

(Short Communication)

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(Received May 18, 1968)

In our previous communication (Mészáros, 1964) we have reported the digestion of ox fibrin with papain and the isolation of glycopeptides. This topic has been the subject of research of several authors (Cynkin, Haschemeyer, 1964; Haschemeyer, Cynkin, 1964; Lipinsky, 1964; Mester et al., 1963, 1965, 1967; Mester, Móczár, 1965). They obtained glycopeptides mainly by digestion with pronase. Haschemeyer et al. (1966) have recently reported the amino acid sequence of three neuraminic acid free fibrin glycopeptides.

The aim of the present work was to determine the amino acid sequence of neuraminic acid containing glycopeptides which were obtained from ox fibrin by digestion with papain and subsequent hydrolysis with pronase.

Experimental

Ox fibrinogen and fibrin were prepared, enzymatically digested and the digests were subjected to Sephadex chromatography by the methods described in our earlier communication. Papain was prepared from papaya-latex and three time recrystallized. Carboxy-peptidase was a commercial preparation. The aldohexoses were determined with orcinol-sulphuric acid (Weimer, Moshin, 1952). Neuraminic acid was detected by means of the Bial reagent (Böhm et al., 1954), amino acids and the other ninhydrin positive substances by means of a 0.1 per cent ninhydrin solution in acetone. The analyses of amino acid and glucosamine were carried out according to Moore and Stein in the amino acid analyzer. The samples for amino acid analysis were hydrolyzed in the usual way with azeotropic hydrochloric acid for 20 hours, the samples for glucosamine determination were treated with 4 N hydrochloric acid for 5 hours at 105 °C. MN 300 G cellulose was used for thin layer chromatography. As a solvent butanol – acetic acid – water mixture (4 : 1 : 1) was applied.

Preparative electrophoresis. Part of the glycopeptide mixture eluted from the Sephadex column was first fractionated in a pyridine acetate buffer (pH 5.6) on a whole sheet of paper in a vertical apparatus in the way described in our previous communication. As the analysis of the amino acids indicated that the three frac-

tions obtained in this way were not yet homogeneous, the fractions were subjected to electrophoresis at pH 3.7. The ninhydrin positive zones were eluted with water. After lyophilization three neuraminic acid containing glycopeptides were obtained. Their compositions are shown in Table 1 (1−1, 1−2, 1−3).

Table 1

Composition and N-terminal residues of glycopeptides

The quantities are expressed as moles with aspartic acid as a unit

	1−1	1−2	1−3	2−1	2−2
Lysine	—	—	—	0.78	0.3
Arginine	0.52	0.50	—	0.66	—
Aspartic acid	1.0	1.0	1.0	1.0	1.0
Threonine	—	—	1.0	—	—
Serine	—	—	0.87	—	—
Glutamic acid	0.82	0.62	—	1.2	1.0
Glycine	—	0.52	—	—	—
Glucosamine	2.76	3.4	3.5	3.1	2.9
N-terminal residue	Glu	Gly	Asp	Glu	Glu

Hydrolysis with pronase. The other part of the glycopeptide mixture which had been obtained by digestion with papain and purification on the Sephadex column was subjected to a second digestion with pronase. The glycopeptide mixture (1 g) in a 0.01 M calcium chloride solution (50 ml) was allowed to stand with pronase (10 mg) at 37 °C (pH 7.9). When alkali consumption has ceased (2−3 hours) the solution was neutralized, concentrated in a rotary evaporator and fractionated on a Sephadex G 25 (40 × 100 mm) column. 345 mg of lyophilized material with 25 per cent aldohexose content was obtained from the carbohydrate-containing fractions. From this fraction the glycopeptides were separated by means of preparative electrophoresis. The compositions are shown in Table 1 (2−1, 2−2).

Determination of the N-terminal residue was performed by the "dansyl"-method (Gray, Hartley, 1963); the DNS amino acids were identified by high-voltage electrophoresis (see Table 1).

Degradation by carboxypeptidase. The glycopeptide 1−3 (0.2 mg) was dissolved in 0.1 M Tris-buffer (50 μl, pH 8) and allowed to stand at 37 °C with carboxypeptidase A which had been prepared according to Fraenkel-Conrat et al. From this solution samples were taken at intervals and subjected directly to thin layer chromatography. The chromatogram of the 30 minute sample showed serine, of the 120 minute sample serine and threonine. After an additional incubation for 24 hours only these amino acids were detectable.

Results

From the amino acid composition, determination of the N-terminal residue and carboxypeptidase degradation and based on other published data (Haschemeyer et al., 1966) the following amino acid sequences of the peptides were obtained:

1–1	Glu-Asp-Arg
1–2	Gly-Glu-Asp-Arg
1–3	Asp-Lys-Thr-Ser
2–1	Glu-Asp-Arg
2–2	Glu-Asp

No experiments were carried out to decide whether Glu is present in the form of glutamic acid or glutamine. The oligosaccharide part is bound to the amide group of asparagine (Mester et al., 1967).

Sequences 1–2 and 2–1 agree with the amino acid sequences of the glycopeptides obtained by Haschemeyer et al. (1966) from neuraminic acid-free ox fibrinogen with pronase and also with the results of Mester et al. (1967).

The author wishes to thank Mrs Kálmán Medzihradszky for performing the analyses of amino acids, Mrs Zoltán Török and Erika V. Varga for their valuable help in the experimental work.

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Effect of Diethylpyrocarbonate on Proteins

II. On the Role of Histidyl Residues in H Meromyosin ATPase

(Short Communication)

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(Received June 21, 1968)

The role of histidyl residues in the activity of myosin ATPase has been the subject of research of many authors. Pelletier and Ouellet (1961) and Hotta et al. (1962) assumed from the effect of pH and Zn^{2+} ions that histidine is an essential component in the centre of myosin responsible for ATPase activity. Stracher and Chan (1964) arrived at similar, though rather inconclusive results in their study of the photooxidation of myosin. They have found that parallel to the loss of enzyme activity in addition to the histidyl residues the cysteinyl and methionyl side-chains were also oxidized. Stracher's recent results (1965) obtained by the photo-oxidation of myosin which had first been subjected to disulfide–sulfhydryl interchange provided more convincing, though indirect evidence. In this case only the histidyl groups were in fact oxidized, the oxidation of methionyl and of the residual 1 SH group (calculated for 200 000 g of myosin) was so slow that this could not be correlated with the ATPase activity decrease measured in the presence of Ca^{2+}.

The application of diethylpyrocarbonate (DEP) offered a new possibility of studying the role of histidine as – according to the results of Mühlrad et al. (1967) and Ovádi et al. (1967) – when used at pH 6 this reagent carbethoxylates specifically only the histidyl residues of the protein. In this way it was possible to block the histidyl residues without affecting the other amino side chains of the protein.

Experimental

The experiments were carried out with H meromyosin as preliminary investigations have shown that myosin precipitates on DEP treatment.

H meromyosin prepared according to Szent-Györgyi (1953) was used in the experiments. The molecular weight of meromyosin was accepted as 362 000 (Young et al. 1964). DEP was a product of Bayer ("Baycovin").

DEP treatment was applied under the following experimental conditions: H meromyosin concentration 2.2×10^{-5} M; DEP concentration 10–160 equiva-

lents calculated for H meromyosin. Treatment was at 0 °C, pH 6 in the presence of 0.05 M sodium acetate buffer. H meromyosin was used 12 hours after the addition of DEP; during this time the excess reagent decomposes completely in the aqueous medium into CO_2 and ethanol (Török et al. 1958).

Table 1 shows the number of side-chains which had been carbethoxylated as a result of DEP treatment. When 160 equivalents of DEP were used practically all the 43 histidyl residues of H meromyosin were carbethoxylated.

Table 1

Formation of carbethoxyhistidine vs. the concentration of DEP and the effect of the treatment on the helix content of H meromyosin

Carbethoxylation was performed at 0 °C in 0.05 M acetate buffer pH 6. The carbethoxyhistidyl residues were estimated according to Ovádi et al. (1967) by means of the differential extinction at 240 mμ ($\Delta\varepsilon = 3.2 \times 10^3$) with a Spektromom 202 photometer. The helix content was calculated according to Simmons et al. (1966) from the rotation value at 233 mμ obtained in optical rotation measurements (between 270 and 225 mμ). The measurements were carried out with an Opton REP spectropolarimeter

$\frac{\text{mole DEP}}{\text{mole of H meromyosin}}$	$\frac{\text{mole carbethoxyhistidine}}{\text{mole H meromyosin}}$	helix content %
0	0	37.5
10	5.5	37.5
20	11.3	37.5
40	17.2	37.5
80	29.2	37.5
120	38.2	28.4
160	42.9	28.6

In the following experiments the changes in the ATPase activity of meromyosin in the presence of EDTA, Ca^{2+} and Mg^{2+}, respectively, were studied as a function of the carbethoxylation of histidine (Fig. 1). The enzyme reaction was stopped by adding trichloroacetic acid to an end-concentration of 5 per cent. The liberated P_i was measured by the method of Fiske and Subbarow (1925). Activity in the presence of EDTA begins to decrease after the blocking of 10 histidyl residues, while after the binding of all histidine residues about 10 per cent of the initial activity is maintained. The activity measured in the presence of Ca^{2+} shows first a mild increase and begins to decrease only after the blocking of 33 histidyl residues. Activity measured in the presence of Mg^{2+} increases considerably as a result of carbethoxylation and will after the blocking of all histidyl residues still be higher than the activity of the untreated control.

The effect of carbethoxylation on the conformation of H meromyosin was studied by measuring the dispersion of optical rotation (Table 1). These results show that only the blocking of the bulk of the histidyl groups brings about some change in conformation. Decrease in helicity (Table 1) appears at the same stage

of carbethoxylation as the decrease in ATPase activity in the presence of Ca^{2+} and Mg^{2+}. It may therefore be assumed that the activity decrease in the latter activities is a result of the conformation change in the molecule. These results are in contradiction with Stracher's findings (1965). He demonstrated a direct

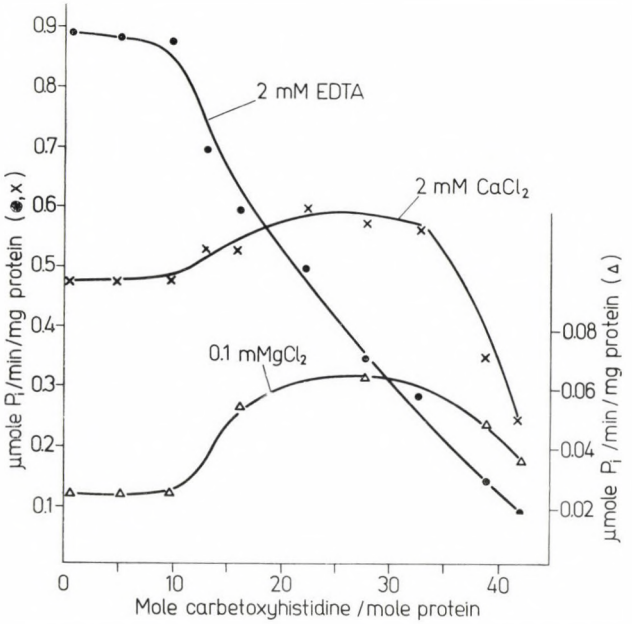

Fig. 1. The effect of carbethoxylation on the ATPase activity of H meromyosin. Experimental conditions of the measurement of enzyme activity: 0.4 mg/ml H meromyosin, Tris—HCl 20 mM, pH 7.2, 20 °C. ● = 2 mM EDTA, × = 2 mM $CaCl_2$, △ = 0.1 mM $MgCl_2$. Ordinate: µmole P_i per mg of H meromyosin per minute. Left: ATPase activity in the presence of Ca^{2+} or EDTA. Right: in the presence of Mg^{2+}

correlation between the oxidation of one histidine group of myosin and the decrease in ATPase activity in the presence of Ca^{2+}.

According to our investigations the decrease in ATPase activity in the presence of EDTA and the increase in activity in the presence of Ca^{2+} or Mg^{2+} occur when about the same number of histidyl residues are reacted. A similar correlation (i.e. that the decrease in EDTA activated ATPase and the increase in Ca^{2+} activated ATPase appear in the same stage of modification) was described for a great variety of amino acid side chain reagents by several authors (Kielley, Bradley, 1956; Kitagawa et al. 1961; Sekine, Kielley, 1964; Fábián, Mühlrad, 1968). The application of an organic solvent, namely of dioxane has a similar effect (Azuma, 1968). It is, however, improbable that the binding of cisteinyl (Kielley, Bradley, 1956; Sekine, Kielley, 1964), or of lysyl (Kitagawa et al., 1961),

or of the histidyl residues (Fig. 1) in the active centre and the application of dioxane would have directly the same effect. It appears more probable that under the action of all these rather different factors the conformation of the active centre of myosin undergoes a change by passing from one thermodynamically possible state into another and this will cause identical changes in activity. Conformation change is limited to a relatively small part of the molecule and cannot be demonstrated by the measurement of optical rotation.

It seems therefore highly probable that the histidyl residues are not constituents of the centre of myosin responsible for enzymic activity but their blocking will affect myosin ATPase through changes in the active conformation.

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Single-column Procedure for the Automatic Analysis of Amino Acids

(Short Communication)

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(Received October 9, 1968)

The method of Spackman et al. (1958) for automatic amino acid analysis was first modified by Piez and Morris (1960) using a single column with gradient elution, and a few years later by Larsen (1965). A Varigrad system and a running-time of 24 hours are required for completing an analysis with both these methods.

We found that by employing the spherical resin Beckman M82 further simplification of the single-column method was possible. Instead of using a gradient elution, the molarity of sodium citrate buffer, pH 4.25 commonly used with amino acid analyzers, was increased. In this way, by the use of two buffers only, all the amino acids present in the hydrolyzate of a protein can be separated from a single sample.

Materials

Apparatus

Beckman Unichrom amino acid analyzers (No. 61001 and 61036) are used in our laboratory. The standard long-column (69×0.9 cm) with Beckman M82 spherical resin (filling height 55 cm) is applied for the single-column procedure. The flow rate of buffer is 100 ml per hour, that of the ninhydrin-reagent is 50 ml per hour. The temperature is 55 °C. The back-pressure of the buffer is about 18 atm. under these conditions.

Chemicals

All chemicals used are analytical grade. Ninhydrin and Methyl Cellosolve are Bio-Rad, all others Reanal (Budapest) products.

Buffers

A) Sodium citrate 0.2 N, pH 3.28. This buffer is identical with that described by Spackman et al. (1958).

B) Sodium citrate, 0.8 N, pH 4.25. The molarity of this buffer is four times higher than that of Spackman et al. (1958).

The ingredients of the buffers are shown in Table 1.

Table 1

Preparation of citrate buffers

Ingredients in 5 liter		A	B
Citric acid monohydrate	g	70.5	282.0
HCl, 37 per cent, density 1.19	ml	61.6	167.5
NaOH	g	40.0	160.0
BRIJ 35	g	10.0	10.0

The quality of distilled water is critical. If it contains ammonia, a plateau is observed between the ammonia and histidine peaks. This is eliminated by using deionized water, obtained from a mixed-bed resin.

Results and Discussion

Figure 1 shows a typical analysis of a synthetic calibration mixture containing 0.25 μmole of each amino acid. The analysis includes the following steps:

a) The elution is started with buffer A similarly to the original, two-column procedure.

b) The second buffer is 0.8 N citrate, pH 4.25 (B) and buffer-change is set to 70 minutes.

c) Run-control is set to 245 minutes.

If the sample analyzed does not contain humins, peptides etc., NaOH treatment of the resin is not necessary and regeneration can be carried out with buffer A. If the sample possibly contains materials not eluted with buffer B, 6 ml 0.2 N NaOH should be fed to the top of the column before regeneration. In both cases regeneration is completed within 30—35 minutes.

The reproducibility, expressed as the maximal deviation of the arithmetic mean is shown in Table 2. The average value is 2.2 per cent, i.e. better than that of the two-column system with Unichrom.

Using the single-column procedure with two buffers, there is a 60-minute period between the elution of histidin and arginine. This can be shortened in two different ways:

a) Using a pH 4.25 sodium citrate buffer with a higher molarity (1.0—1.2 N). In this case the interval between ammonia and lysin is smaller, the separation is less satisfactory since a double peak is formed when ammonia is present in a great excess.

b) Using a third buffer, 1.05 N sodium citrate, pH 5.28.

The second buffer-change is set to 150 minutes, i.e. following the elution of ammonia. Since the automatic control of buffer-change in a standard Unichrom analyzer only allows one buffer-change, the three-buffer system can only be used with partly manual control.

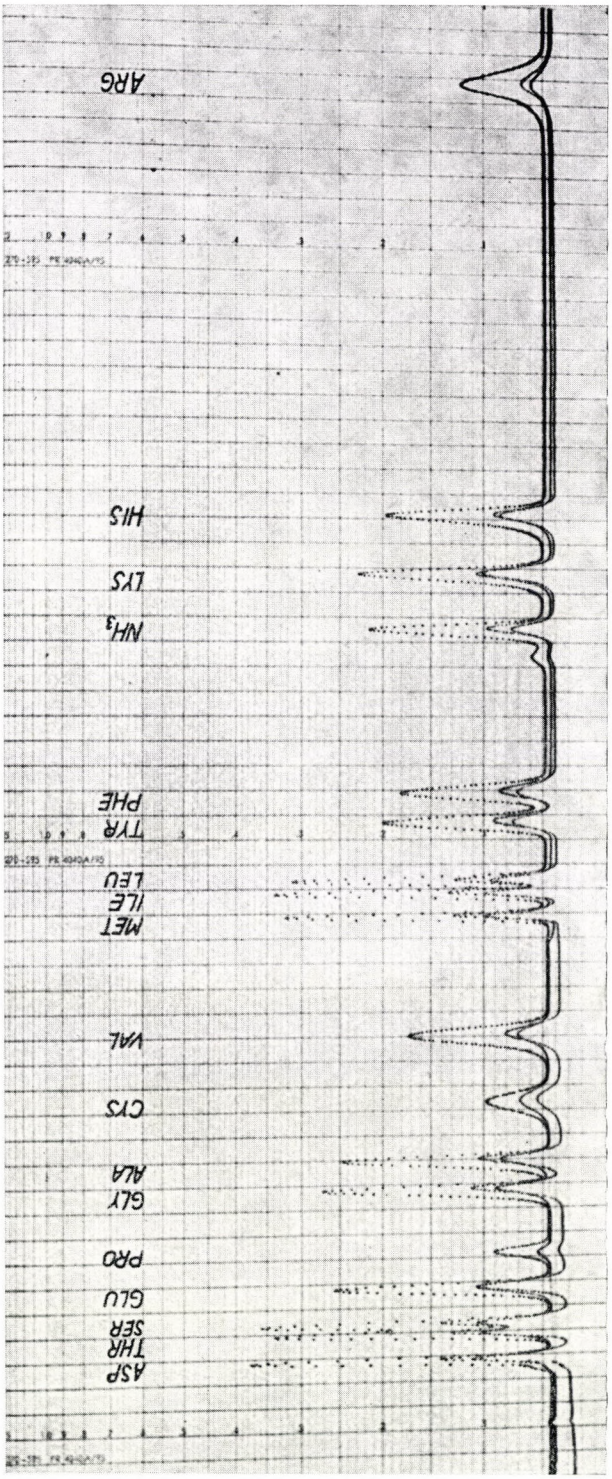

Fig. 1. Fractionation of a synthetic mixture of amino acids on a single column, with the two-buffer system

Table 2

Integration constants obtained from three analyses of a calibration mixture
The analysis was carried out with 0.25 μM of each amino acid. The calculated figures are given for 1 μM

	148/5	150/5	155/5	Arithm. mean	Maximal deviation from the mean %
ASP	19.96	20.0	20.4	20.12	0.8
THR	22.02	22.4	22.49	22.63	1.7
SER	23.12	23.7	23.04	23.28	1.8
GLU	20.58	19.52	20.24	20.11	3.0
PRO	4.32	3.05	4.22	4.19	3.4
GLY	21.28	21.28	20.94	21.16	1.1
ALA	21.40	20.84	21.30	21.18	1.7
CYS	10.12	9.84	9.28	9.74	4.8
VAL	21.28	20.88	20.52	20.89	1.8
ILE	19.95	20.08	18.60	19.5	4.9
LEU	20.70	20.80	19.81	20.43	3.1
TYR	19.08	18.72	18.92	18.90	1.0
PHE	18.84	18.56	18.68	18.69	0.8
LYS	22.12	22.32	21.45	21.96	2.4
HIS	19.95	20.16	19.47	19.86	2.0
ARG	18.57	18.20	18.12	18.29	1.6
				Average:	2.2

One sample is sufficient for the single-column analysis by the two-buffer system. This gives a fast and easy way to separate all the amino acids present in the acid hydrolyzate of proteins or in physiological fluids within 4 hours without any special equipment, with any type of a standard amino acid analyzer.

The author would like to thank Dr Helmuth Fellenzer for samples of the recently developed spherical resin Beckman M82.

The technical assistance of Mrs Judith Báti is gratefully acknowledged.

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Ultrastructure of Myofibrils after Selective Protein Extraction

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The ultrastructural changes caused by selective protein extraction were investigated. It can be concluded that the basic filamentous system of myofibrils is given by the protein fibrillin. This filamentary system of about 40 Å in diameter runs through the whole sarcomere and shows a highly elastic character. The actin-containing filaments start from the Z line and show a sheave-like arrangement between the traversing filaments.

The location by protein extraction of the structure proteins in isolated myofibrils has been investigated in details by Huxley and Hanson with the interference microscope (Huxley, Hanson, 1960). The development of preparative methods especially that of fixation, embedding and thin sectioning has made possible the extracted substances to be examined with electron microscope (e.g. Garamvölgyi, Kerner, 1966).

In biochemical experiments minced muscle or isolated myofibrils and for electron microscopy small samples with a well-defined orientation are used. The relative amount of solvents and the time of extraction must be changed according to the requirements. Electron microscopic examinations on selectively extracted samples have shown that after the removal of myosin and actin there remained a continuous filamentary system connecting the Z lines (Guba, 1968; Guba et al., 1964).

The protein of this system was isolated and characterized physico-chemically (Guba et al., 1968). The protein was named fibrillin which refers to its role in the basic filamentary system.

The present work describes the changes in the ultrastructure of myofibrils during selective protein extraction. In addition, some conclusions are drawn as to the location of proteins in the myofibrillar structure.

Materials and Methods

In the experiments fresh m. psoas, glycerinated m. psoas and isolated myofibrils of rabbit were used. Care was taken to maintain adequate cooling (0—5 °C). 20—30 mm long small bundles with a diameter of 1—3 mm were cut from the muscle and fastened to wooden sticks.

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The extraction of the proteins was performed with 100 volumes of the corresponding solvent for different periods of time under magnetic stirring.

1. Solvents

a) Hasselbach-Schneider solution: 0.47 M KCl, 0.01 M $Na_4P_2O_7$, 0.01 M KH_2PO_4, pH 6.1–6.3

b) ATP-ascorbic acid solution: 0.2 mM ATP, 0.2 mM ascorbic acid, pH 7.0–7.4

c) 50 mM $NaHCO_3$

d) KI solution: 1.0 M KI, 0.01 M $Na_2S_2O_3$, 0.01 M Na_2HPO_4, 0.01 M ATP, pH 7.5

The reagents were of analytical grade and were produced by the Firm Reanal (Hungary).

2. Preparation for electron microscopy

A double fixation consisting of 2 hours in a 3 per cent solution of glutaraldehyde (pH 7.2–7.4) followed by 1 hour in a 2 per cent solution of OsO_4 (pH 7.0–7.2) was used. Dehydration was performed in a graded series of ethanol-water mixtures and embedding in an Epon mixture.

Sections of 150–350 Å in thickness were cut with a home made and with a Sorvall Porter Blum (MT-2) ultramicrotome using glass knives. The sections were stained either in Pb-citrate according to Reynolds (1963), or a double staining with UO_2-acetate and Pb-acetate was used. Examinations were carried out by means of a Hitachi HU-10 electron microscope at 75 kV on Agfa Diapositiv Hart plates.

Magnification was calibrated by polystyrene particles with a diameter of 1880 Å.

3. Detection of the proteins

The extraction of myosin was checked by determining the ATPase activity of the extracted protein and of the residue. For protecting the enzyme the solvents used contained 10^{-4}M of β-mercapto-ethanol. The remaining enzyme activity was checked in the samples fastened to the sticks and in the pooled samples removed from the sticks. No essential difference seemed to be between the results of the two measurements.

To control the removal of actin (after it had been washed with $NaHCO_3$ solution and distilled water) an acetone dry powder was made from the residue in the usual way and the protein extracted from this was investigated. (The removal of actin from the muscle structure seems to be very difficult, some traces of actin remaining even after repeated extractions.

Results

The effect of protein extraction on the myofibrillar structure has always been related to that of the control.

Fig. 1 shows the well-known structure of a control myofibril on a cross section in relaxed state. In Fig. 2 the same is demonstrated on a longitudinal section.

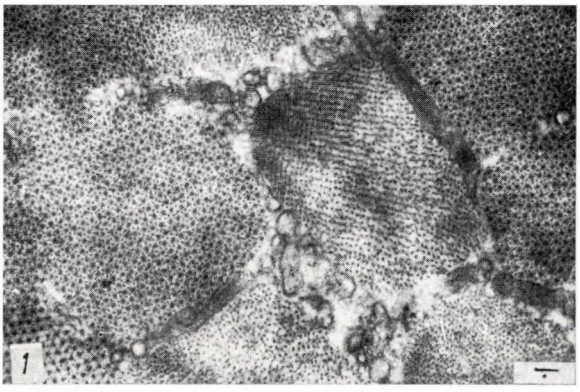

Fig. 1. Cross section of an untreated relaxed myofibril (control)

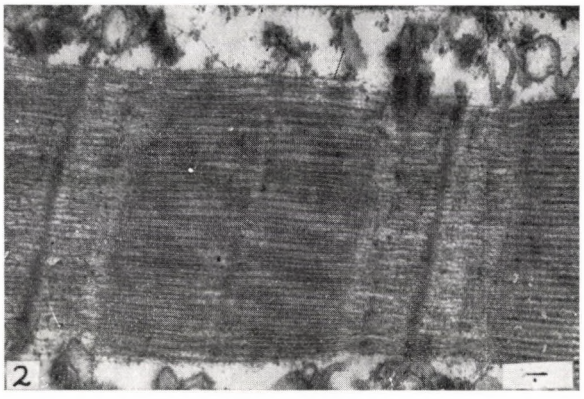

Fig. 2. Longitudinal section of an untreated relaxed myofibril (control)

The observations on the ultrastructure of untreated myofibrils have been published elsewhere (Guba et al, 1965; Guba et al., 1968a)

1. The effect of myosin extraction

Table 1 contains the data on myosin extraction with Hasselbach-Schneider solution. Myosin gives about 38 per cent of the total amount of muscle proteins. With progressive myosin extraction the electron scattering power of the A band

Table 1

Data on myosin extraction

Protein extraction	Amount of myosin extracted in per cent of the total protein content	Myosin left in the system (in per cent of total myosin content)
H—S I.	31.5	17.0
H—S II.	5.0	4.0
H—S III.	1.0	1.0
H—S IV.	0.5	0.0
Total:	38.0	—

gradually decreases, and the primary and secondary filaments cannot be distinguished any more. After the extraction of myosin a sheave-like formation and also a continuous filamentary system can be observed (Fig. 3). The lengths of

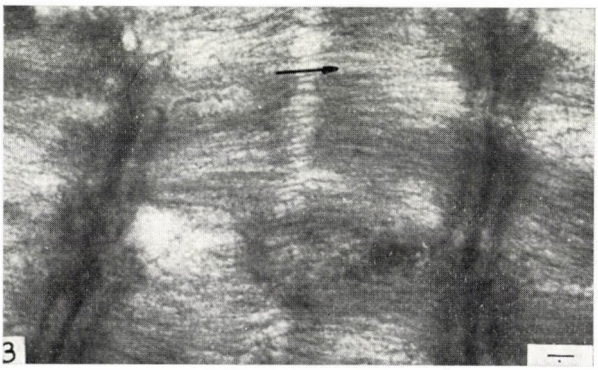

Fig. 3. After myosin extraction a sheave-like formation and a continuous filamentary system can be seen

the "sheaves" show a constant value of about 1.2 μ. In some cases a cross periodicity of 300—400 Å can be observed (indicated). The "sheaves" are separated from each other by the previous H band. The size of this part varies with the length of the sarcomere and seems to contain a highly elastic component (Fig. 4). The "sheaves" are fastened by the Z lines.

It seems that there exist two types of filaments: the one is continuous and the other terminates at the edges of the "sheaves".

The micrographs on cross sections of myofibrils show the decomposition of the double filamentary system and the hexagonal arrangement even at the very beginning of myosin extraction (Fig. 5).

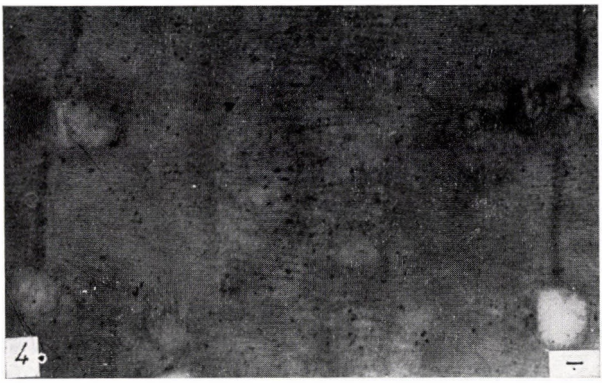

Fig. 4. The bundles of the "sheaves" are separated by the previous H band. The length of this part varies with the sarcomere length and seems to contain an elastic component

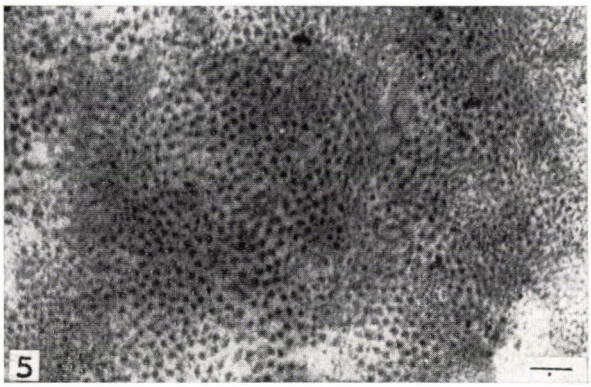

Fig. 5. Disorganization of the hexagonal pattern and indistinctness of filament sizes after myosin extraction

Further extraction results in a change of the filament's size. The primary filaments become thinner but they keep their original thicknesses in the middle part of the sarcomere (previously H band). Here, the number of filaments is 650 SD 50 in an area of 1 μ^2 and the distance between them is 400 SD 50 Å.

The diameter of the filaments varies between 100 Å and 170 Å. The number of thinner filaments in an area of 1 μ^2 is 2000 SD 200. Their arrangement resembles the structure of the A–I border in the control. The diameter of the filaments is about 60 Å.

2. Effect of the extraction of actin

Table 2 contains the data on the amount of the extracted and residual actin. The whole actin content of the muscle is about 15 per cent.

Table 2

Data on actin extraction

Protein extraction	Amount of actin extracted (in per cent of the total protein content)	Actin left in the system (in per cent of total actin content)
ATP-ascorbic acid blending I	3.0	80.0
ATP-ascorbic acid blending II	8.0	27.0
0.1 M KCl	1.0	20.0
Total	12.0	20.0

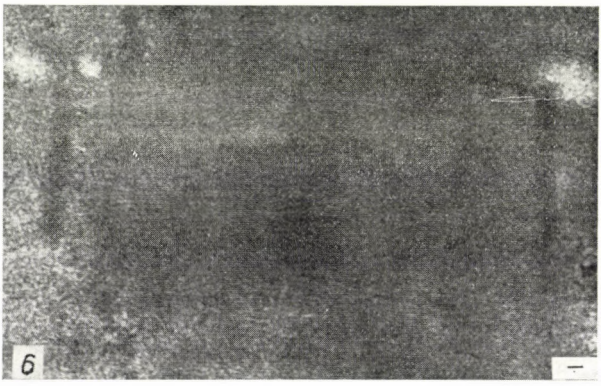

Fig. 6. After complete removal of actin a continuous system remains consisting of filaments with a diameter of about 40 Å. The sign on the micrographs indicates 0.1 μ

Partial or complete removal of actin does not result in disorganization of the filamentary structure; a further decrease is, however, observed in the electron density.

After the complete removal of actin there remains a continuous system consisting of extremely thin filaments with a diameter of about 40 Å (Fig. 6).

3. The extraction of fibrillin

After a further protein extraction with a solution of KI the organized structure has been fully destroyed.

Discussion

The extraction may result in a change of the rearrangement of the proteins because of the disruption of their original structure. Therefore, one has to be very careful in making any statement as to the location of proteins. Thus, our examinations are of comparative character. It is to be noted that the untreated material has a high protein content filling the whole space available (Guba et al., 1965). In the course of extractions the protein content of the system diminishes; therefore, a change is likely to occur in the spatial arrangement.

The differences between the proteins building up the filamentary systems must also be taken into account. Bearing in mind the considerations mentioned above we can draw the conclusions as follows:

1. The extraction of proteins results in a gradual decrease in the electron density of the whole myofibrillar structure and does not lead to abrupt disappearance of any distinct structural detail. This indicates that the proteins build up the structure in a tightly bounded and complex way.

2. The removal of myosin causes the hexagonal arrangement to undergo disorganization at the very beginning of the extractions. This seems to prove that the hexagonal arrangement in the untreated muscle is a consequence of the close packing of myofibrillar proteins.

In accordance with known data (e.g. Corsi et al., 1967) the contrast of the A band decreases and the central part of the sarcomere (previously H band) contains the relatively smallest amount of material. This part shows a high degree of elasticity.

The sheave-like structure with the Z line in the centre shows great similarity to the isolated I segments (Huxley, 1963) and seems to exhibit a constant length.

If the primary filaments were built up exclusively of myosin the primary filaments should be missing in the micrographs after the removal of this protein. Nevertheless, the micrographs show the cross sections of "primary" filaments, though with a thinner diameter, even after myosin had been extracted. The distance between the filaments is larger in the extracted than in the untreated samples. The pattern of filaments seems to change from hexagonal into a more or less tetragonal form. As it has been shown the diameter of the filaments in the central part of the sarcomere seems to be enlarged. Consequently, the extraction of myosin does not result in the disappearance of the primary filaments; they become, however, looser and thinner and their density diminishes.

3. The removal of actin does not abolish the filamentary structure, a uniform system remaining running through the whole sarcomere. The density shows a further decrease. The determination of the filament's number in a given unit area becomes very uncertain. Further experiments are needed for accurate determinations.

The staining conditions seem to support the view that fibrillin gives the core of the primary filaments.

This possibility would agree with the continuity of the filaments (Garamvölgyi, 1965).

4. The filamentous system is completely destroyed after the extraction of fibrillin.

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Size of the Filaments in Relaxation and Contraction

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The ultrastructure of relaxed and contracted rabbit m. psoas myofibrils was investigated using adequate cross sections. Measurements were performed as to the size of the filaments and as to the distance between the primary filaments in relaxed and contracted myofibrils. A change was found in the size and relative number of the filaments during contraction. It is suggested that the functional changes of the myofibril are based on modification of its gel structure.

Introduction

Low angle X-ray diffraction studies performed on living myofibrils (Elliott et al., 1963, 1967; Huxley, Brown, 1967) indicated a hexagonal lattice with a spacing of 350–450 Å for the primary, myosin-containing filaments. The series of low angle reflections observed in different physiological states of the muscle have provided valuable informations concerning the detailed helical arrangement of the molecules in the primary and secondary filaments. Electron microscopy has visualized the double array of thick and thin filaments. Nevertheless the continuity of the filamentary systems seems to be an important problem of muscle research. Hypotheses are based on the discontinuity (Huxley, Hanson, 1960) or on the continuity (Garamvölgyi, 1964–65, 1965; Guba, 1966) of the primary and secondary filaments.

Materials and Methods

The experiments were performed on the m. psoas of the rabbit. Thin bundles of standard length were used. Contraction was induced in a solution containing 5×10^{-2} M KCl, 10^{-3} M $MgCl_2$, 5×10^{-3} M ATP at pH 7. Contraction of the bundles was allowed to occur up to about 2/3 of their resting lengths. Double fixation: solutions of 3 per cent glutar-aldehyde and 1 per cent OsO_4 were used.

Embedding was accomplished in an Epon-812 mixture. Sections were cut on a Porter-Blum MT-2 ultramicrotome with glass knives. The sections were stained either according to Reynolds (Reynolds, 1963) or a double UO_2-acetate and Pb-acetate staining was used.

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Electron microscope examinations were carried out with a Hitachi HU-10 apparatus at 75 kV accelerating voltage on Agfa "Diapositiv Hart" plates.

Magnification was calibrated by standard polystyrene spheres of 1880 Å.

The measurements on the micrographs were made with an Abbe type (Zeiss) comparator microscope.

Results

Myofibrils in relaxation

Fig. 1 shows the structure of the A band. The primary filaments appear as having no definite outlines. The centre of the thick filaments shows a decreased electron stain affinity resembling to that of insect flight muscle. This part is surrounded by subfilaments varying in number between 4 to 7 (arrow). In some cases the secondary filaments show a subfilamentar arrangement too (double arrow).

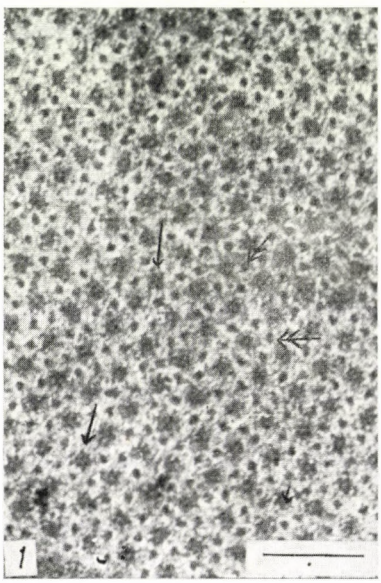

Fig. 1. The structure of the primary and secondary filaments in the A band of relaxed myofibrils

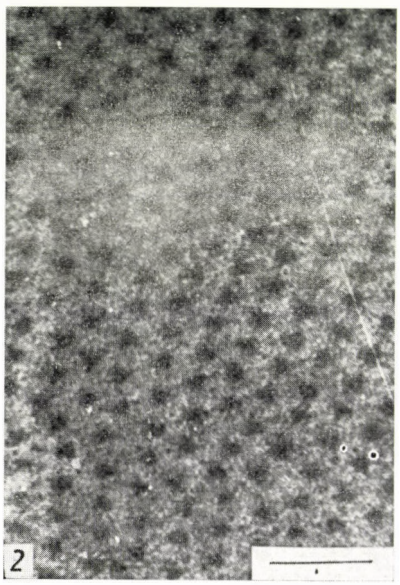

Fig. 2. Cross section at the H band. Thin threads branch out from the primary filaments

Fig. 2 shows the structure of the H-band. Thin threads branch out from the primary filaments and a network structure is seen.

Fig. 3 demonstrates the A–I junction. The hexagonal arrangement clearly shows up even far from the zone containing the well-defined primary filaments (indicated). The primary filaments seem to continue in the I band too, even though in a thinner form (indicated). The system reaching the Z line is transformed into a tetragonal arrangement (Fig. 4).

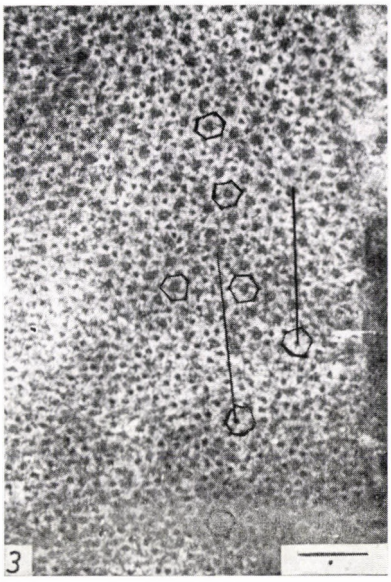

Fig. 3. Micrograph showing the A—I junction. The primary filaments seem to continue in the A band too

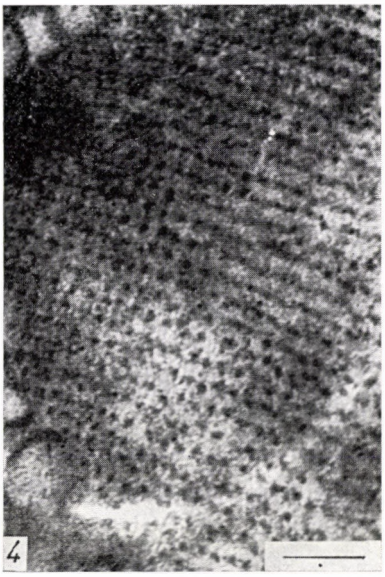

Fig. 4. Near the Z line the arrangement transforms to a tetragonal one

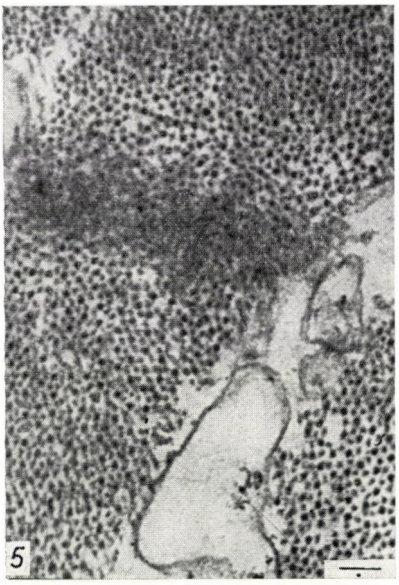

Fig. 5. The arrangement of a shortened myofibril near the contraction band at the Z line (C_Z)

Myofibrils in contraction

Fig. 5 demonstrates a sarcomere near the C_Z contraction band. The primary filaments became thinner and the secondary ones thicker as compared with relaxed myofibrils. Fig. 6 shows the structure near the C_M band. The relative number of thick and thin filaments shows a great variety (indicated). Fig. 7 shows that the

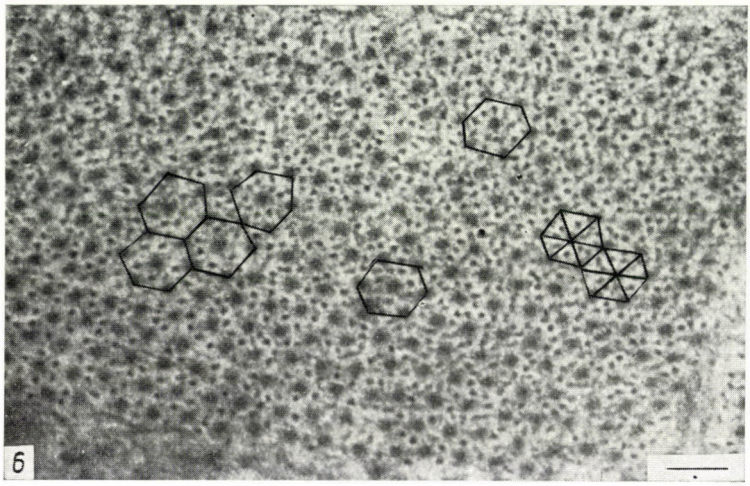

Fig. 6. The structure near the contraction band at the M line (C_M)

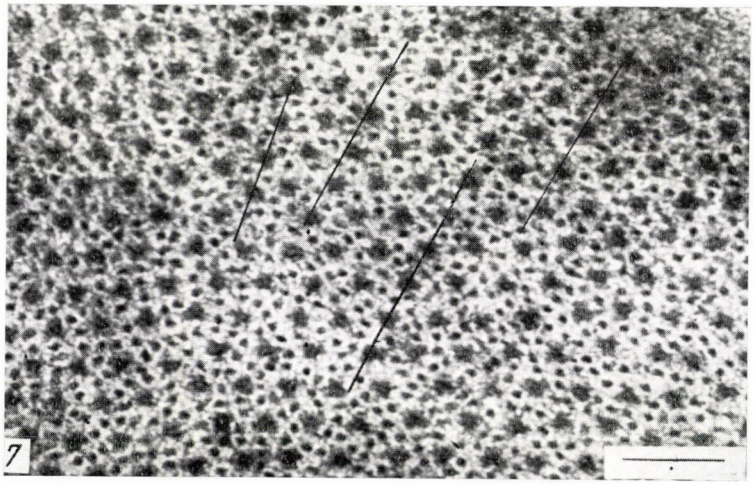

Fig. 7. The hexagonal pattern of the primary filaments is less regular. "Dislocations" indicated. The sign on the micrographs indicates 0.1 μ

Table 1

Data on the size of the filaments in the relaxed muscle

	H		A		A – I junction		I	
	Å	SD	Å	SD	Å	SD	Å	SD
Lattice constant (distance of the primary filaments)	360	20	360	18				
Diameter of the primary filaments	160	21	120	24	70	24		
Diameter of the secondary filaments			65	4	65	10	80	10

The number of filaments was calculated for 1 μ^2 area.

Table 2

Number of filaments in 1 μ^2 area in the relaxed muscle at different parts of the sarcomere

	A		A – I junction		I		Z	
	n/μ^2	SD	n/μ^2	SD	n/μ^2	SD	n/μ^2	SD
PF	1045	32	—	—	—	—	—	—
SF	2100	46	—	—	—	—	—	—
F	—	—	2800	253	2300	215	2400	420

The area occupied by the myofibrils was found to be 92±2 per cent of the fibre.

Table 3

Data on contracted myofibrils

	"H"		"A" at H		"A" at C	
	Å	SD	Å	SD	Å	SD
Lattice constant (distance of the primary filaments)	415	30	—	—	360	21
PF ∅	200	12	—	—	115	8
SF ∅	—	—	50	10	90	7
SF (number): Pf (number)?			Rate 2.8	SD 0.5	Rate 2.0	SD 0.1

The area occupied by the myofibrils was found to be 72 ± 15 per cent of the fibre.

hexagonal pattern of the primary filaments is not regular, in the "crystalline" structure dislocations appear (indicated). Data of 12–12 micrographs obtained on cross sections of 5 relaxed and 5 contracted myofibrils were measured. The data on lattice constant and on the size of the filaments in the relaxed state are summarized in Table 1. The number of filaments in an area of 1 μ^2 in the relaxed muscle at different parts of the sarcomere is demonstrated in Table 2. The area occupied by the myofibrils in the relaxed state was found to be 92 $\pm$ 2 per cent.

The data on muscle in the contracted state are shown in Table 3. The area occupied by the myofibrils in this case was found to be 72 $\pm$ 15 per cent.

Discussion

The preparation for electron microscopy: fixation and embedding denature and precipitate the myofibrillar proteins. Application of glutaraldehyde seems to be superior in this respect to other fixatives used previously. Cross sections investigated in electron microscopy are suitable to perform quantitative measurements (Guba et al., 1965).

By electron microscopy we found a lattice constant of 360 SD 18 Å. This seems to agree with the data obtained by X-ray diffraction (Huxley, Brown, 1967), and is higher than that of Carlsen et al. (1961). According to our data the A band contains about 1000 primary and 2000 secondary filaments in an area of 1 μ^2 i.e. a total of 3000 filaments. This number changes into 2300–2400 in the I band near the Z-line. This is more than the number of the secondary filaments in the A band, but less than the sum of the two kinds of filaments. Nevertheless taking the SD values into consideration, the uncertainty of the counting increases in the direction of the Z line. The sum of the mean value and the upper limit nearly gives the whole filament number of the A band. This means we have to assume the continuity of the primary filaments through the I band too. The quantity of the protein aggregated in the primary filaments is less in the I band than in the A band.

When a straight line is drawn through the spots representing the primary filaments of the A band this can be continued in the I band too, and the hexagonal arrangement is seen (Fig. 3). The primary filaments show a subfilamentar arrangement which was studied in details by Baccetti (1965). The tubular arrangement of the thick filaments is in agreement with our results obtained by selective extraction of the myofibrillar proteins, demonstrating the core of the filaments to consist of a substance with a low electron stain affinity (Guba et al., 1964). The primary filaments are connected to the network system of the M line. Similar observations were described by Pepe (1967).

The secondary filaments seem to start from the tetragonal network of the Z line. They are thicker in the I band than in the A band, and they become very thin or they break in the H band.

The lattice constant of the myofibrils in contraction varies in the different parts of the sarcomere: it decreases from the centre towards the edges with more than 10 per cent. Measurements are easier on contracted myofibrils indicating

a more compact structure of the substances. The diameter of the primary filaments is about twice as large in the centre as in the edges of the sarcomere. The secondary filaments show just an opposite change.

The relative number of two of the primary and secondary filaments is characteristic of the relaxed muscle in the A band. This number increases during contraction in the central part over two but it is not well defined.

The data obtained for the size and number of filaments in the unit area allow us to make some calculations on the protein content represented by the filamentary systems.

Let us accept the myofibrils to give about 60 per cent of the muscle fibre and the proteins to have a density of 1.3 g/ccm. According to the calculations the primary filaments give 43 per cent and the secondary filaments give 34 per cent of the structure proteins in the relaxed state. If a comparison is made between the data mentioned above and the quantity given for myosin and actin, the primary filaments contain about 10 per cent less protein than the amount of myosin. At the same time the secondary filaments contain 10–15 per cent more protein than the amount of actin. Similar calculations for protein content of contracted myofibrils give a value of 65 per cent for the primary filaments and 28 per cent for the secondary filaments of the structure proteins.

These values are, of course, not absolutely quantitative but rather approaching data. Nevertheless the calculations concerning the two physiological states of the muscle indicate an important fact: the state of aggregation of the protein system is different. The relaxed muscle is a looser and a very hydrated protein system. In contraction the protein structure becomes more compact because of dehydration. During preparation for electron microscopy more protein is precipitated and becomes suitable for observation.

Calculations on the space requirement of the myosin molecules support this view. Myosin under physiological conditions forms a gel system of high water content consisting of well-ordered and aggregated molecules. In case the primary filaments are the centres of myosin aggregation this loose gel structure shows a diameter of 420–450 Å in the relaxed state. The gel fills the whole A band and this seems to be the reason for the hexagonal arrangement in the A band, representing the most complete packing. Since in the I band the protein content is less the hexagonal array is not compulsory and it rather shows a tetragonal arrangement.

Following conclusions can be drawn:

1. The primary filaments are continuous.

2. The substance observed in the electron microscope is the precipitated part of the proteins.

3. During contraction the same fixation causes precipitation of a more compact protein structure due to the change in the state of aggregation and in the rate of hydration.

4. According to our measurements myofibrils show a volume decrease of about 4–5 per cent during contraction.

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Contents

Investigations on the Absorption and Fluorescence of Chlorophylls

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1. Absorption and fluorescence of chlorophylls in algal suspensions

a) The action spectrum of fluorescence begins to drop towards longer waves at a wavelength of 680, 654 and 637 nm in Chlorella, Porphyridium and Anacystis, respectively. This phenomenon is due to the presence in the in vivo systems of the two main components of chlorophylls. The investigations show the applicability of the modified Stepanov equation for the determination of the action spectrum of in vivo systems too (1, 2).

b) The artifact introduced by the "sieve-effect" in obtaining the true absorption spectrum of suspensions with highly absorbing particles was eliminated by ultrasonic treatment. It was shown that the low absorption of the Soret-band in Chlorella is due to the flattening caused by sieve-effect. Since the structure of the red absorption band is not affected by sonication the two main components of chlorophylls are present also after sonication (3).

The action spectrum of fluorescence in sonicated algal suspensions highly depends on the pH and on the presence of air. In Chlorella suspensions the red drop of the action spectrum of fluorescence in alkaline medium (pH = 7.8) begins at about 680 nm and the same is found in both aerobically and anaerobically sonicated suspensions. In acidic medium (pH = 4.65) the red drop begins at much longer waves (705 nm) in sonicated suspensions; in this case mainly the long wave component of chlorophyll-a is destroyed by sonication (4).

c) Allowing an error of 2 per cent the spectral effect of secondary fluorescence in chlorophyll solutions can be neglected up to c.d = 10^{-5} mole cm/l the effect of secondary fluorescence on the intensity of fluorescence is negligible up to c.d = 10^{-6} mole cm/l (c and d are the concentration and the layer thickness of the solution). From the saturation of the secondary fluorescence an absolute maximum spectral effect of 5—6 per cent is estimated for the fluorescence and 18 per cent for the quantum yield of fluorescence in chloroplast (5).

2. Energy migration between chlorophylls and other molecules

a) The self-quenching and self-depolarization of fluorescence in chlorophyll solutions show a much more effective migration of electron-exciting energy among chlorophylls than expected assuming an inductive resonance mechanism with a $1/R^6$ distance-law after Förster. Similarly, the foreign quenching of fluorescence of trypaflavin with rhodamine-B seems to be more effective than that which could be explained by a Förster-mechanism. The dependence of the foreign quenching and sensitisation of fluorescence on the exciting wavelength shows the existence of a long range energy migration with a distance law other than $1/R^6$.

b) The "blue" fluorescence of chlorophyll found in in vivo systems was detected also in cyclohexanol solution. The spectral distribution of the emission due to a transition from the second singlet state to the ground state was measured. The absolute quantum yield of this fluorescence is estimated to be about 10^{-3}.

3. Miscellaneous

a) The effect of the spectral composition of light on the chlorophyll and carotenoid content of bean leaves was studied under field conditions. The preliminary results are to be confirmed under controlled conditions in phytotrone (6).

b) The absorption spectra of chlorophyll-a and chlorophyll-b in 19 solvents showed very slight (12 and 15 nm) shift of the location of the red maximum; the extinction coefficient exhibited 40 and 90 per cent changes. The maximum difference of 50 per cent in the oscillator strength is mainly due to physical effects.

c) The aging of bean and mustard leaves was studied by means of the absorption spectrum, emission spectrum, the degree of polarization of fluorescence and the time course of fluorescence. All these characteristics showed a dependence on the age of the leaf and render possible to draw conclusions on the condition of the pigment system (7).

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7) Chlorophyll Photobiology of Aging Bean Leaves in vivo. This meeting (With Aly Raafat, J. Gausz, L. Szalay, I. Horváth)

Effect of Gravity on the Morphology and Metabolism of Plants

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Gravity has a considerable effect on living organisms; higher gravitational fields (up to 5 g) stimulate growth; on klinostats, however, the stimulated zero gravity reduced growth processes.

Experimental plants (Avena sativa L., Lupinus albus L.) were horizontally displaced since germination and turned with 180° each day for 4 weeks. Control plants grew vertically. Basal metabolism (respiration, carbohydrate and nitrogen fractions) and auxin balance have been studied in treated and control plants. The differences found in auxin metabolism may explain the reduced growth.

Photodynamic Influence on Photoinduced Movements in Tetrahymena pyriformis LG

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The individuals of the axenically well-cultivable Tetrahymena pyriformis LG strain are photosensitive. When illuminated by suitable monochromatic light of a high-pressure mercury lamp (200 W) viz. a xenon lamp (500 W) the animal exerts gradually accelerating movement which in 5 to 12 in general, reaches the maximum minutes depending on the wavelength and intensity of the light. This maximum is followed by a transitory decrease of speed and by a lower second maximum; then, again, by a decline in the speed of the movement. The phenomenon is more expressed after permeation of photodynamic dyes (Eosin Y, Janus-green B, methylen-blue, rosa bengale, toluidin blue).

The electron microscopic pictures call attention to the damage of cell constituents, and to that of mitochondria in particular, playing an important part in the electron transport. This certainly exerts an influence on the respiratory ATP synthesis and, as a consequence, on the movement. But, at the first maximum, the light of higher energy shows a relative

refractory period which gradually disappears. The disappearance is shown by the fact that the energy-rich light stimulus is capable of increasing the speed at any part of the descending branch of the speed versus time curve and even of producing a new maximum probably due to the rearrangement of a long-duration excited (triplet) state.

Some Aspects of Oxidation-Reduction Changes in Chloroplasts During the Process of Aging

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The process of aging as well as the phenomena of juvenility and senescence have attracted the interest of many investigators in the recent years. Changes in the structure, chlorophyll content and photosynthetic effectiveness of chloroplasts during aging were reported, yet without data on the underlying oxidation-reduction processes. In the present study we found that in chloroplast suspensions of primary bean leaves the changes in the oxidation-reduction potential (ORP) during periodic illumination show characteristic features depending on the age of the leaf. These changes seem to be related to the chlorophyll concentration of the chloroplasts and to some of the photochemical activities, e.g. Hill-reaction, measured by reduction of DCIP. By adding oxidation-reduction reagents affecting the pigments of systems 1 and 2 conclusions can be drawn concerning the electrochemical mechanism and the sequence of changes in the ORP during periodic illumination. It could be stated that the ORP of samples of different age, starting from nearly the same value ($E_h = 385$ mV $\pm$ 10 mV), increases during the period of illumination and decreases in the dark. These changes are less regular in the case of chloroplasts of young leaves. The overall trend of the ORP curves is to decrease with successive illumination periods. This may be attributed to the exhaustion of the redox capacity of the system which is more obvious in young leaves than in old ones.

Chlorophyll Photobiology of Aging Bean Leaves in vivo

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In order to clarify some aspects of juvenility and senescence experiments were carried out to determine the absorption spectrum, fluorescence spectrum and the degree of fluorescence polarization as a function of the age of primary bean leaves. The total absorption with a peak at 678 nm is lower in young expanding leaves, it increases to a maximum followed then by a gradual decrease in senescent leaves. The half band width and the relative area of the red band behave similarly. Both the intensity and degree of fluorescence polarization increase with the age (e.g. p increased from 0.193 up to about 0.29). The changes with age in absorption and emission spectra can be interpreted by assuming a preferential loss of one of the chlorophyll components of the photosynthetic systems. The increase of the degree of polarization can be attributed to a less effective energy transfer due to a decrease by aging of the pigment content.

Temperature Dependence of the Relationship between the Absorption and the Emission Spectra of Chlorophyll *a* and its Derivates

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The relationship between the absorption and the emission spectra given by the Stepanov equation, at 298 °K and 77 °K, was studied in the solution of chlorophyll *a*, pheophytin *a* and chlorin e_6 in EPA (ether isopenthan alcohol). The local temperatures T* (of the excited molecule and its surroundings) depend on the nature of the fluorescing substance, frequency of the exciting light and the experimental temperature. While T* was in good agreement with the ambient temperature at 298 °K, it was comparatively much higher at 77 °K. The deviation at 77 °K was maximum in the case of chlorin e_6 and minimum in the case of chlorophyll *a*. Non-dissipation of the excess energy of the excited molecule to the solvent molecules before the act of emission was probably responsible for the higher values of T* at 77 °K. In the case of chlorophyll *a* F(ν) indicated the presence of more than one fluorescent species at 77 °K. This conclusion is in agreement with that drawn by matrix analysis. In the case of pheophytin *a* and chlorin e_6, in which the Mg atom was replaced by H atoms, there seemed to be no indication of more than one fluorescent species. The dependence of T* on the excitation energy in excess to that of 0—0 transition could possibly be the reason for its variation with the frequency of the exciting light.

On the Role of Stimulus Threshold of Some Microreceptors in Information Coding

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Sense organs can be generally interpreted also as sets of elementary microreceptors. A characteristic threshold-value called resting threshold is coordinated to each microreceptor. These threshold-values follow a probability distribution pattern. It is a known fact that a microreceptor reacts with firing only when reached by an adequate external stimulus corresponding to, or excelling, the threshold value. This provides the sense organ i.e. the microreceptor with varying amounts of information depending on the intensity of the stimulus and the length of time.

The firing frequency depends on the intensity of the external stimulus and on the length of time. Mathematically one can approximately describe how the stimulus threshold changes in time from the beginning of firing. This is called instantaneous threshold. With the help of this function we can explain the experimental fact that by changing the stimulus intensity also the frequency of the excitatory impulse burst is changed, i.e. according to which mathematical regularity is the change of the amount of information coded into an excitatory impulse burst by the instantaneous threshold of the microreceptors.

Naturally, the function describing the instantaneous threshold change of the microreceptor does not give further information about the complicated physico-chemical (biochemical) changes which are characteristic of the metabolism of microreceptors during that period and which essentially determine the entire functional process.

* On leave from the Institute of Experimental Physics, József Attila University, Szeged, Hungary.

Ultrastructure of the Striated Muscle and Moiré Patterns

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Electron micrographs of the striated muscle display great variations. It is a principle of submicrostructural investigation of this Institute that a two dimensional picture is not identical with the three dimensional structure. For this reason investigations were carried out to search the origin of these patterns. Certain ultrastructures are in good agreement with moiré patterns: *a)* the so called pseudo-striation observable on the muscle fibril; *b)* the periodic thickening observable on the filaments; and *c)* the series of thick and thin filaments following one another. Phenomena similar to those which can be seen in electron micrographs were produced by models as moiré patterns. At the same time it was shown that, on the ground of these patterns, a possibility is given also for quantitative evaluation concerning the ultrastructure of the muscle.

Structure of the Muscle-Fibril

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The solution of this problem is indispensable for an understanding of the function, and it requires many-sided investigations. In connection with the question of how the fibril is held together mechanically, this lecture deals with three points: 1) is there a fibrolemma; 2) are the longitudinal structural elements passing continuously through the Z-discs; 3) are there any cross-formations beside the Z, H, M, N, formations more or less generally accepted?

Mechanical and Structural Relations of the Bee Flight Muscle, I

J. BELÁGYI, N. GARAMVÖLGYI

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Due to the lack of sarcolemma, the minute amount of serial and parallel elastic components and its great extensibility, the flight muscle of bee represents a very suitable object to study the mechanical properties of the muscle.

The resting tension–length diagram — apart from the stress relaxation — exhibits a characteristic change at about 100 per cent extension which can be very well interpreted on structural basis. The active isometric tension shows a highly asymmetric course in relation to the length, and it decreases to zero at a length corresponding to the characteristic change obtained in the resting tension–length diagram. On the basis of the mechanical properties even extreme stretches are reversible.

Mechanical and Structural Relations of the Bee Flight Muscle, II

N. GARAMVÖLGYI, J. BELÁGYI

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The mechanical properties demonstrated in the previous paper may be brought in connection with the changes observed by us in the cross-striation. Neither the length – tension diagram of the resting muscle, nor the reversibility of extreme degree elongation can be interpreted on the basis of the sliding theory based on a discontinuity of the filaments, but difficulties arise in the case of the assumption of a simple continuity, too. The relation "active isometric tension—length" differs from the similar results obtained on vertebrate muscle. This difference corresponds to the specific structural conditions of the insect flight muscle. All our data are conform with our sarcomere model constructed earlier which could be characterized as a "coiling and sliding" model in order to distinguish it from the classical "sliding" model.

Autoradiographic Investigation on the Localisation of K in the Cross-Striated Muscle and the Effect of Direct Stimulation on the Intrafibrillar $^{39}K-^{42}K$ Exchange

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The experiments were performed in order to establish the intrafibrillar K distribution in cross-striated muscle by electron microscopic and light microscopic autoradiography. Simultaneously with the investigations into localisation, data were obtained on the role of direct stimulation in $^{39}K—^{42}K$ exchange.

Frog's (Rana esculenta) Läwen-Trendelenburg preparation was perfused with a Ringer solution containing ^{42}K isotop. Two preparations were perfused simultaneously and one of them was stimulated directly with 50 Hz electric current at a frequency of 30/min till fatigue. Further investigations were performed with the sartorius muscles of the two frogs. As photographic material Ilford L–4 liquid emulsion was used.

The experiments revealed the ^{42}K radioactive isotop to be suitable for autoradiography. It was established that 65.5 per cent of the grains originating from ^{42}K is located over the A-band and 34.5 per cent over the I-band which contains also the Z-line. Finally was shown that the direct stimulation significantly increased the intrafibrillar $^{39}K—^{42}K$ exchange ($P = 0.02$).

On the Mechanism of Plaque's Development

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One of the oldest methods used to detect bacteriophages is the one based on evaluation of plaques caused by phages. It is generally accepted that development and growth of plaques is a rather complex process which seems yet to be unexplained in many respects. A method has been developed for the examination of plaque-growth. With the aid of this method which

seems to be more exact than the previous ones we were able to demonstrate that the growth-curves of coliphages T7 plaques behave step-like in phase II. By increasing the concentration of host bacterium microcolonies by two orders of magnitude only a slight decrease can be observed in both the height and width of the steps. But none of these quantities shows a significant dependence on the age of the plaques. These phenomena can be interpreted by presuming, beside diffusion and adsorption phenomena, also the existence of an interaction between phages and bacteria (e.g. pole-dipole) up to the distance of several microns. This interaction results in the development of a cloud of phages around the bacteria. A lower limit was given for the range of action and the diameter of phage-clouds was estimated.

On the Development of the Linear Phase of Plaque Growth

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The increase in diameter of plaques of T7 coli phages was studied in the linear (IInd) phase as a function of the incubation time. The average rate of increase characteristic of the phase II of plaque's development, was found to be 0.77 ± 0.0078 cm/h. We determined the extrapolated axis sections belonging to the 0 plaque-diameters. It has been established that the distribution of the axis sections has a complex nature. Investigations were carried out on the dependence of this distribution *a)*, on the concentration of bacteria originally poured into the Petri dish, *b)* on the antecedents of the indicator bacteria (synchronous, asynchronous), as well as *c)* on the condition of the phages producing the plaque (untreated, irradiated with UV for 2.10 sec). On the basis of the data obtained indirect conclusions can be drawn as to the properties of the phage-bacterium complex involved in the primary infection (radiation injury of phages, host cell reactivation, etc.).

On the Intracellular Phage Development

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The burst size of phages T7 has been determined in E. coli B host cells. The phages have been subjected to an UV-light irradiation for 0, 2 and 10 seconds, respectively. As a consequence of irradiation the average burst size decreases, the values are 147.2; 86.8; and 96.2, respectively. The distribution of the burst size can be divided into two components: in the case of a 2 second irradiation 60 per cent of the original population remains undamaged and 15 per cent becomes damaged (the remaining 25 per cent is not capable of provoking an infection). In the case of 10 second irradiation these ratios are 18 per cent, 9 per cent and 73 per cent, respectively. According to our previous results the second part of the population is formed by phages damaged by irradiation but reactivated in the host cells. Our models for the intracellular phage-development are based on the above experimental results and can be summarized as follows. Let number ζ denote a quantity which characterizes in Model A the precursor material of the phages and in Model B the whole energy and row material of the cell, and let η be the number of ready phages. In Model A from the state (ζ, η) two transitions are possible, namely to

the states $(\zeta+1, \eta)$ and $(\zeta-1, \eta+1)$. In Model B from the state (ζ, η) steps may occur to the states $(\zeta-1, \eta)$ and $(\zeta, \eta+1)$. Model A is based on the fact that the infection started a new process in the host cell which continues untill the quantity of ready phages reaches the level of precursor material. In Model B the phage production simply uses up the reserves of the cell.

Uptake of Antibiotics by Bacteria

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We have studied the uptake of streptomycin by E. coli B in relation to the incubation time by using different streptomycin concentrations.

Three phases can be distinguished on the curves resulted from our experiments. In the first phase lasting a few minutes the quantity of streptomycin taken up quickly increases, followed then by hardly any further change for about half an hour (plateau phase). In the third phase the streptomycin uptake increases again. We have concluded that the antibiotic molecules are bound first to the cellular membrane and only later can some of them get into the interior of the bacteria.

As an explication for the formation of plateau phase we can suppose a reversible reaction (binding — disjunction, adsorption — desorption) to take place between the binding places on the membrane (molecular ranges) and the antibiotic molecules, which results then in the formation of an equilibrium state. We have interpreted the kinetics of the processes and determined the equilibrium constant of the interaction between cellular membrane and antibiotics.

The third phase of the curves gives information on the speed of the accumulation of streptomycin molecules in the intracellular space. The penetration constant relating to the streptomycin was determined on the basis of this phase.

Change in the K-Content of in vivo Muscles Due to the Effect of Ionizing Radiation

J. TIGYI

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On the basis of earlier data of this Institute and of the literature it has been established that the decrease of potassium content is one of the fundamental reactions of the isolated muscle to irradiation. Within certain dose ranges, the loss of potassium in the muscle is proportional to the given dose.

The data of literature do not agree concerning the in vivo experiments; some research workers, e.g. Wilde and Sheppard did not observe any decrease of potassium content in the isolated rat muscle even after irradiation at 73 kr.

The present investigations have shown that one can observe, even after irradiation with doses smaller than lethal, significant decrease of the K-content. The decrease in the K-content may even reach 30 per cent in the case of a lethal dose (DL_{50}).

The results are discussed in relation to the development of radiation diseases and besides, from the point of view of the basic phenomena of radiation effects.

Effect of Trace Elements on the Scintillation of Biological Substances

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In 1966 we reported observations on scintillation induced in biological substances by incorporated tritium β-radiation. The increasing effect of trace elements on radiation sensitivity in biological substances is known from the literature (Skok, 1957; Eyster, 1958; Crane, 1958). Niedetzky has shown in this Institute (1963) their effect on radiation sensibility of frog hearts. In the present experiments the effect on this scintillation of some trace elements has been investigated.

In the experiments sartorius muscles of Rana esculenta were incubated in Ringer solution containing tritium (25 μCi/ml) and, except the control, also trace elements in a concentration 10^{-5} to 10^{-3} M at 2 °C for 24 to 72 hours. The number of scintillations was registered with a photomultiplier. The bivalent Cu ions were found to be especially effective, and they resulted in an increase of the number of scintillations almost one order of magnitude over the control. Thus, the efficiency of scintillation/β-decay reaches a few decimal per cent. The same ions do not increase the scintillation of actomyosin solution, and also the univalent Cu ions have a different effect on the muscle. A slighter effect was observed also with others ions such as Sn^{++}, Mg^{++} and Ag^{+}.

The results suggest that the excitations and a possible semiconductor mechanism caused by the impurity of trace elements may play a part in the primary radiation effect.

Effect of Soft Beta Radiation on the Excitation of Muscle

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At the last years's meeting we presented data on the effect of the beta radiation of tritium (18 KeV max. energy) on the excitatory process of the muscle. It was established that, when compared with untreated control, the stimulation threshold of the irradiated muscle decreased after a few thousand rad absorbed doses.

In the present work the changes in submaximal action potentials due to tritium irradiation were investigated on isolated frog sartorii incubated at a temperature of 2 °C. Submaximal action potential could be evoked for an 80 to 100 per cent longer period of time on muscles kept in a Ringer solution of 10 mCi/ml specific activity than on muscles kept in normal Ringer solution under similar conditions.

Highest effectiveness was seen at 10 mCi/ml activity out of the investigated ranges of 0.5—5.0—10—15—20—50 mCi/ml, i.e. at 600 to 700 rad/day.

The aforesaid data can be evaluated as positive radiation effect, similarly to other experiments of the same character performed in this Institute earlier.

Effect of γ-Radiation on the Lifetime of Sperms

A. NIEDETZKY

Biophysical Institute, Medical University, Pécs

Isolated, mature frog's sperms were irradiated by different doses of ^{60}Co γ-radiation in physiological solution. After irradiation the number of moving sperms was determined from time to time, and expressed in relative terms compared with the initial value. Non-irradiated suspensions from the same preparation served as control. The number of moving sperms decreased roughly exponentially with the time. In 43 experiments with doses under 1000 rad no significant difference has been found in the time necessary to reduce by 50 per cent the number of moving sperms in control and irradiated specimens. This period decreased by more than 50 per cent in the case of doses over 1000 rad. As it had been reported earlier an equal decrease in motility could be achieved even by β-radiation with doses which were one order of magnitude lower than those used in the present experiments.

The Effect of Ionizing Radiation on the Copper Metabolism of the Plasma

B. KANYÁR, J. NAGY

Biophysical Institute, Medical University, Budapest

We studied the distribution of ^{64}Cu isotope in various organs of rats subjected to 200 and 500 rad whole body irradiation. The isotope was administered by the intraperitoneal route in form of $CuSO_4$ 24 hours after irradiation. 6, 17 and 28 hours after administration of the isotopes the animals were decapitated and the ^{64}Cu content of various organs was determined. 6 hours after irradiation the femur showed a decreased and the kidneys an increased activity when compared with controls. In the 17 and 28 hour experiments the plasma activity of the irradiated animals significantly increased, which can be explained by an increased ceruloplasmin level. ^{64}Cu-plasma (ceruloplasmin) labelled in vivo in donor animals was injected into control and irradiated rats, and the decrease of their plasma activity was studied. The whole body doses employed by us did not alter the speed of ceruloplasmin consumption: the biological half-time of the disappearance of the labelled plasma was 22 ± 3 hours.

Initial Volume Decrease of Muscle as Excitation Phenomenon

F. ARADI

Biophysical Institute, Medical University, Pécs

Initial volume decrease of muscle has been investigated on frog's ischiadicus-gastrocnemius preparation in tensionless state of indirectly stimulated muscle. The measurements were carried out by a piezoelectric pressure transducer used by Ernst and coworkers earlier. The experimental data have shown that *a)*: In the case of single stimulus the duration of initial volume decrease is commensurable with the duration of action potential; *b)* Under

the effect of stimulus series the volume decrease shows high periodicity just as does the action potential; *c)* Single stimulus is not always followed by single volume decrease but in some cases by an oscillating volume decrease as it had been, concerning, the course of action potential, too. Our experimental results support the conception that, in contrast to recent data in the literature the initial volume decrease of muscle is characteristic of the proceeding phase of mechanical activity, i.e. of the excitatory state.

Frequent Excitation of the Motor Nerve, I

P. VADON

Biophysical Institute, Medical University, Pécs

Experiments were performed to investigate the production of frequent excitation in nerve sections soaked in Ringer solution containing glycerine. The action potentials of the nerve and the muscle contractions were registered simultaneously on frog sciatic nerve–gastrocnemius muscle preparation. When the soaked nerve section was stimulated by a square pulse of 20 V amplitude and of *0.1 ms* duration a series of action potentials could be conducted from the not soaked part of the nerve, and the muscle contraction had a tetanic course. These experimental results support the conception that also motor nerves are capable of reacting by frequent excitation to *one single short stimulus.*

Frequent Excitation of the Motor Nerve, II

G. BÍRÓ

Biophysical Institute, Medical University, Pécs

Frequent excitation generated by the motor nerve has been investigated on frog's ischiadicus-gastrocnemius preparation with simultaneous registration of the nerve action potential and the muscle contraction. When the portion of the nerve soaked in Ca-free Ringer solution was stimulated by a rectangular pulse of 20 V and *0.1 ms* in duration frequent action potentials could be recorded on the nerve, and the muscle contraction was of the tetanus type. The experimental results refer to the ability of the motor nerves to respond with frequent excitation to *one short stimulus* too, and so these nerves could be considered as "active channels".

Excited Electrons and Nerve Excitation

T. LAKATOS

Biophysical Institute, Medical University, Pécs

In our experiments action potentials were produced by intense illumination with visible light in the sciatic nerve of the frog sensitized by Na-eosin, neutral red or bengal rose. Action potentials were observed only during illumination. The isolated nerves were put into Ringer solution containing one of the dyes in a concentration of 10^{-5} to 10^{-3} g/ml; and were kept there in total darkness for 60 to 120 minutes. The illumination was performed by a tungsten filament lamp through a water layer in order to eliminate infrared rays. The intensity of illumination was 80 000 lux. Action potentials were registered by a cathode-ray oscilloscope. The results of the experiments can be explained by supposing the light-excited electrons of the dyes to play a part in the generation of nerve excitation.

On the Problems of Modelling Local Potentials in Nerve Fibres

L. KIRÁLYFALVI

Biophysical Institute, Medical University, Pécs

The impulse-generating system of the nerve fibre or muscle fibre can be modelled in the general case by an active electronic system. It should be pointed out that, in contrast with previous interpretations, an active electrical system is necessary also for modelling the linear local potentials in general case. There are namely such forms of local potentials which support the participation of active gain elements. — A transistorized model has been constructed, suitable for demonstrating these relationships. Together with subthreshold potential records taken for comparison from the literature the responses of this system to the small-signal input are shown.

Interpretation by Information Theory of Subthreshold Stimulus Summation

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The state of excitation capable biological systems will be characterized by the relative frequency function of the stimulus force and by the value of the stimulus threshold. Thereafter, starting with these parameters, the information theoretical entropy is used to define the state of the system.

Depending on the strength of the stimulus every single stimulus modifies the state of the system and, at the same time the entropy characteristic of it. In this manner every stimulus is interpretable by the entropy decrease caused by it i.e. by the amount of information carried by it.

Applying the outlined approach and concepts one can formulate the phenomenon of summation quantitatively. The relative frequency function of stimulus force being explained on the basis of speculative considerations, the obtained result can be turned into a form which provides the possibility to analyse the whole chain of thought experimentally.

Finally attention is called to some possible generalizations.

Transfer Function of a Geometrical Neuronal Configuration of the Cerebellar Granular Layer

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An attempt is made to analyse the transfer function of the neuronal information preprocessing system of the cerebellar granular layer.

First the geometrical model of the regular neuronal arrangement is presented. Then the transfer function of the model is analyzed and illustrated by a pattern transformation method.

In conclusion it can be stated that the granular layer owing to its basic functional and structural features (i.e. its local averaging procedure and the structural redundancy) may have an error suppressing role increasing the reliability of the mossy fiber input channel of the cerebellum.

Investigation of the Water Binding of Protein Solutions by Microwave Measurements

G. MASSZI

Biophysical Institute, Medical University, Pécs

According to several concepts in the literature the water bound to the protein has an "ice structure", and its amount is 0.2 to 0.4 g water/g protein. Since the dielectric constant at 3 GHz of the ice and protein is small ($\varepsilon < 5$) one would expect the microwave dielectric constant and conductivity of the protein solutions to decrease approximately linearly with increasing concentration. On the contrary, according to the measurements performed on gelatine solutions, the change of the dielectric constant differs from linearity even at 20 to 30 per cent concentration level, and the microwave conductivity of the solutions increases with the concentration. The results cannot be interpreted by hydration in the above mentioned sense of individual molecules. The observation can be explained, by interaction of different molecules on one hand, and by migration of charge in the close vicinity of the protein molecule on the other.

The Structure of Bound Water in Muscle

S. PÓCSIK

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The existence of bound water in protein is generally accepted in the literature. The structure of bound water is considered ice-like, the density of which is lower than 1. According to our experiments in which the mass and volume of the muscle was measured the density of muscle water is greater than 1. Performing the experiments below zero °C, we obtained further informations concerning the structure of bound water.

Data on the Mechanism of Osmoregulation in Unicellular Organisms

F. VETŐ

Biophysical Institute, Medical University, Pécs

According to Lovtrup and Pigon, as well as Kitching the concentration of the freshwater Protozoa's plasm is about 50—100 mOsm, while the concentration of the medium is only about 7 mOsm. To explain this phenomenon it is necessary to know the quantitative relations of osmoregulation. 1. The frequency of the Paramaecium's pulsating vacuoles decreases exponentially when the concentration of Ringer solutions is increased from 0 mOsm to 240 mOsm. The deviation considerably increases in the case of solutions over 100 mOsm, a fact indicating the cardinal importance of this concentration. 2. In sucrose solutions the frequency of vacuoles in animals paralyzed by 25 mg per cent of $Ni(NH_4)_2(SO_4)_2$ is independent of the concentration in the interval of 0—60 and 60—100 mOsm. There is, however, a sudden decrease in the frequency at about 60 mOsm. 3. The frequencies of the two vacuoles differ, the one in the vicinity of the nucleus is 10 per cent slower. 4×10^{-5} M dinitrophenol increases the frequency by 12 per cent. The nature of the problem and the data available support the possibility of the thermoosmosis.

Separation of Isotopes by Thermodiffusion

P. VARGA-MÁNYI

Biophysical Institute, Medical University, Pécs

After separating ^{39}K and ^{42}K as well as ^{40}Ca and ^{45}Ca isotopes we report at present on the separation of ^{1}H and ^{3}H isotopes in a model planned and constructed by us. This is a three-wall glass vessel with reserve volumes on its lower and upper part. The working space of the thermodiffusion tube was filled with redistilled water containing tritium. The efficiency of separation caused by the effect of temperature gradient was evaluated by the separation factor (2): in the majority of the experiments the separation factor relating to the activity was: $\frac{a_{\text{lower}}}{a_{\text{upper}}} > 1$.

Study of AET Metabolism by Labelled Compound

L. SZTANYIK, A. SÁNTHA, E. MÁNDI

"Frederic Juliot-Curie" National Research Institute for Radiobiology and Radiohygiene, Budapest

The absorption, distribution and transformation of radioprotective compounds in the organism as well as their excretion rate directly determine the optimum conditions for their application. At the same time, the revealing of the nature of their metabolic feature promotes also the elucidation of the mechanism of radioprotective action.

In the experiments reported the metabolism of ^{14}C—AET was studied in mice following intraperitoneal or oral administration. The concentration changes of the compound were determined as a function of time in the blood, various tissues and excreta of the animals. It has been established that the duration of the radioprotective effect of AET reflects the changes of the tissular concentrations of the compound.

On the Biological Effects of Incorporated Radioactive and Stable Isotopes

I. The Effect of Inorganic ^{32}P on the Development of Chick Embryo

G. J. KÖTELES, L. D. SZABÓ, F. ANTONI

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The effect on the viability and development of chick embryo of various doses of inorganic ^{32}P injected after various incubation periods was studied. When given at the third day of embryonic development, 200, 100 and 50 μCi per egg resulted in a 50 per cent mortality within 1, 4 and 10 days, respectively. 25 μCi per egg, however, did not cause any higher mortality rate than that of the controls until hatching. Definite retardation of growth always preceded the death of embryos. In order to try to differentiate between radiation and trans-

mutation effects the frequency of ^{32}P in nucleic acids was determined on the one hand, and, on the other, model experiments applying chemical dosimeter were performed to measure the radiation doses absorbed by the embryo body.

On the Biological Effects of Incorporated Radioactive and Stable Isotopes

II. The Effect of Transmutation of ^{32}P Incorporated Into Ribonucleic Acids on the Protein Synthesis

L. D. SZABÓ, J. HOLLAND, G. J. KÖTELES, E. BÖLÖNI, F. ANTONI

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The effect of transmutation of ^{32}P incorporated *in vivo* into RNAs of chick embryo liver was studied on the cell-free *in vitro* protein synthesis. Applying various labelling conditions ribosome preparations were obtained from embryonic liver which contained 5—20 nCi ^{32}P per 100 μg RNA—P. Such preparations were tested for their amino acid incorporating ability immediately after isolation and after storage at —20 °C for one and two physical half-lives of ^{32}P. It has been found that, depending on the number of decayed ^{32}P atoms within the RNA molecules of ribosomes, the incorporation of ^{14}C-amino acids decreased. Furthermore, the changes in the acceptor functions of ^{32}P-labelled transfer RNAs were studied under the same experimental conditions (specific activity and storage). The transmutation of intramolecular ^{32}P caused a decrease also in this function of transfer RNAs. This decrease proved to be different for the various transfer RNAs carrying various amino acids even in the case of the same ^{32}P activity.

On the Biological Effects of Incorporated Radioactive and Stable Isotopes

III. The Effect of Heavy Water on the Amino Acid Incorporation of Isolated Liver Microsomes

J. HOLLAND, L. D. SZABÓ, F. ANTONI

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In the presence of $^{2}H_2O$, depending on the concentration, the ^{14}C-amino acid incorporating activity of microsomes isolated from guinea-pig liver increased significantly. The degree of increase depends also on the content of Mg^{++} and $^{2}H^{+}$ ions of medium. This increased functional activity could be observed in the presence of both endogenous or exogenous messenger RNA. However, it never occurred if the ribosome fraction was prepared from the microsomes by deoxycholate treatment and tested under similar conditions. The distribution according to sedimentation of microsomal fractions showing increased ability for amino acid incorporation was analyzed by sucrose density gradient ultracentrifugation. Experiments were performed to reveal the intermediary step of protein synthesis that might be involved into the increased functional activity. The species-specificity of the phenomenon was also studied. Our investigations suggest that heavy water influences the conformational state of a regulatory factor acting at the microsomal level.

Investigation on the Interaction between Uranium and Various Amino Acids, and Determination of the Stability Constants of their Complexes

M. Csővári, G. Lichner

Ore Mining Company Mecsek, Health Service, Pécs

It was the purpose of these investigations to study the distribution of uranium in the organism and to facilitate the search for substances promoting the excretion of uranium from the organism. For this purpose the investigation on the interaction of uranium with proteins, nucleic acids, amino acids, hormones, etc. and the study of the stability of their complexes may yield the necessary informations. In the present work we report on the complex formation between uranium and some amino acids playing a highly important part in the composition of proteins. The stability constants of the mononuclear complexes of uranium with 20 amino acids have been determined by means of ion-exchange resin and calculated by the method of Fronaeus. Great care was taken to avoid any uncontrolled hydrolysis of the complexes during the reaction.

Investigation on the Trace Element Content of Whole Human Blood by Neutron Activation Analysis

T. Zsoldos

County Hospital, Industrial Hygiene Service,
Low-Level Activity Laboratory, Pécs

The dust content of the lungs of miners increases with the time spent in the mine. Trace elements present in the dust get into the metabolism of the organism by chemical and biological solubilization. Their detection, similarly to cases of intoxication, is possible by the analysis of the excreta or the blood.

We have chosen some trace elements present in the dust causing exposition, and followed the changes of their blood concentration for several years from the beginning of the exposition. These data are planned to serve as a basis for drawing conclusions by mathematical modelling as to the dust charging of the lungs.

In order to have appropriate basis for comparison we have analysed the chosen trace elements in blood samples taken from 20 healthy men, who never worked in mine.

Evaluation was performed by taking up the gamma spectra of the irradiated blood with a semi-conductor detector and by analysing them after radiochemical separation with a multichannel amplitude-analyser connected to a NaI(Tl) crystal detector. Simultaneously we investigated the solvent effect of blood plasma with regard the ore-dust causing exposition.

Some Problems of Investigating Iodine-Kinetics

K. G. Bartha, B. Kanyár

Biophysical Institute, Medical University, Budapest

The iodine kinetics of the organism can adequately be studied on the 131-iodine metabolism of the thyroid gland as well as on the 131-iodine excretion from the blood. The three rate coefficients α, η, σ characterize the iodine metabolism. The rate coefficient α is charac-

teristic of the uptake of iodine by the thyroid, σ of the secretion of hormonal, organically bound iodine from the thyroid and η of the iodine excretion from the iodine distribution space. These rate constants are easily calculated by means of the iodine turnover and blood excretion curves on the ground of the following equation:

$$P_m = \frac{\alpha}{[\alpha + \eta]\,\sigma}\,[e^{-\sigma t} - e^{-(\alpha+\eta)t}]$$

where $P_m = {}^{131}\mathrm{I}$ contained in the thyroid gland.

To calculate the rate constants reliably the points of measuring should be determined with a satisfactory accuracy. It was our aim to eliminate the stress effect unfavourably influencing the iodine metabolism caused by tying of the animals during the experiments. The animals were put untied into a plexi-cylinder. This circumstance influenced the constant geometry of measurement unfavourably. So we had to use a lead diaphragm of large diameter. As a consequence of using the large diaphragm even the impulses coming from the environment were already measured, a fact distorting the data especially those obtained a few hours following the administration of iodine. To avoid this source of error we corrected the numbers of impulses detected above the thyroid gland. The simultaneous determination of the curves of iodine storage and excretion renders possible the rate constants characteristic of metabolism to be calculated not from the uptake period of the curve but from the mobilization period and the blood resorption curve, which both can be determined more exactly.

Changes in Plasma Haemoglobin Concentration after Ionizing Radiation

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"Frederic Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene, Budapest; and Health Service of the Hungarian People's Army, Budapest

Ionizing radiation decreases the resistance of circulating red blood cells to different damaging influences. The following partial or complete destruction of the fragile cells may be assessed most accurately by measuring the increase of the plasma haemoglobin level. We examined the fragility of red blood cells in blood samples taken from patients receiving X-ray therapy as well as from persons occupationally exposed to ionizing radiation. The samples were treated *in vitro* by ultrasound of different intensities and the results were compared with the values obtained in healthy individuals under identical conditions We found that following both X-ray therapy and occupational low-level exposure the basic plasma haemoglobin concentration was considerably increased when compared with control values, and these differences were further accentuated by ultrasound treatment. Increased fragility of the red blood cells in individuals exposed to ionizing radiation may be explained by the changes in the composition of erythrocyte population. Some extremely high values obtained during the first 24 hours of X-ray therapy indicate the assertion of direct radiation effects on the blood.

Application of Media in Animal Dosimetry

I. Nikl

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For the purpose of a more exact determination of the radiation dose and dose distribution as well as for standardization of irradiations in radiobiological experiments performed on animals with external sources animal media should be used for the measurement of the dose.

The author reports on such media developed by him: phantom, prepared and fresh animal cadavers, and *in vivo* media. Reference is made to some data obtained with measurements performed on such media.

Some Aspects of the Conditions for Measurements of Radiation Protection and their Application in Practice

K. DÓSAY

"Frederic Joliot-Curie" National Institute for Radiobiology and Radiohygiene, Budapest

The elucidation of the problems of radiation protection or the explanation of their settlement have to be supported by results of measurements. The author emphasizes the importance of the radiation protection measurements and the necessity of ensuring certain prerequisites. The energy dependence characteristics of two different types of ionization chambers, taken up with a practically homogeneous X-radiation (homogeneity index 1), are reported in connection with the solution of a given problem of radiation protection. The author is of the opinion that unanimity is the most important feature as regards the determination of energy dependence. On the other hand, the energy dependence data acknowledged to be ideal (taken up with monoenergetic sources) cannot be applied, without essential reduction of accuracy, to settle all the practical problems. Characteristic pictures are projected on the useful modification of the conditions for radiation protection of the "Medicor" UV—2 diagnostic X-ray apparatus.

A Method to Measure Surface Dose by Large Surface Dosimeter

I. BOJTOR

"Frederic Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene, Budapest

The measurement of the dose absorbed by the body is a basic endeavour of dosimetry. In most cases this is realized in an indirect way. The author reports a method using film for measurement. An approaching procedure, i.e. graphic integration is used to evaluate the method. The absorbed dose is calculated from the obtained surface entry dose by making use of the absorption factors of ionizing radiation. The method is applied for determination of the radiation exposure of the population.

Investigations on the Velocity and the Absorptivity of Ultrasonic Energy in Lipids

G. NAGY-GERECZE

Institute of Medical Physics, Medical University, Debrecen

The velocity and the absorptivity of ultrasonic vibrations measured in different media are related to the molecular configuration of the matter. Both data have been measured in some biological media, but definitive conclusions as to the nature of molecular configuration could not be drawn from the results achieved so far.

In the course of the experiments investigations were carried out with the help of the measuring method developed by the author on the temperature depending changes of the velocity and absorptivity of ultrasonic energy in lipids.

Using her own results and those of the literature, the author studies the relationship between the above mentioned two physical parameters and the molecular configuration of the lipids.

Effect of ^{60}Co Gamma Rays on the Tryptic Digestion of Phosphorylase b

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As is known from earlier investigations the allosteric properties and feed-back sensitivity of some enzymes are far more sensitive to ionizing radiation than any other functional properties of the macromolecules.

The present paper deals with the tryptic digestibility of phosphorylase *b* (EC. 2.4.1.1.) in the presence and absence of the allosteric effectors of the phosphorylase. AMP, ATP and G—6—P all inhibited — depending on their quantity — the tryptic digestion of phosphorylase *b*. Since the allosteric effectors did not affect the tryptic digestion of casein, we suggest an indirect mechanism for the above-described phenomenon.

The ^{60}Co-irradiation of phosphorylase *b* greatly decreased the protective effect of AMP, ATP and G—6—P on tryptic digestion. The digestibility of control phosphorylase was not altered by the irradiation dose.

The results confirm our earlier suggestion that the radiation sensitivity of the allosteric regulation — i.e. the fine molecular transitions — is higher than that of other properties.

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