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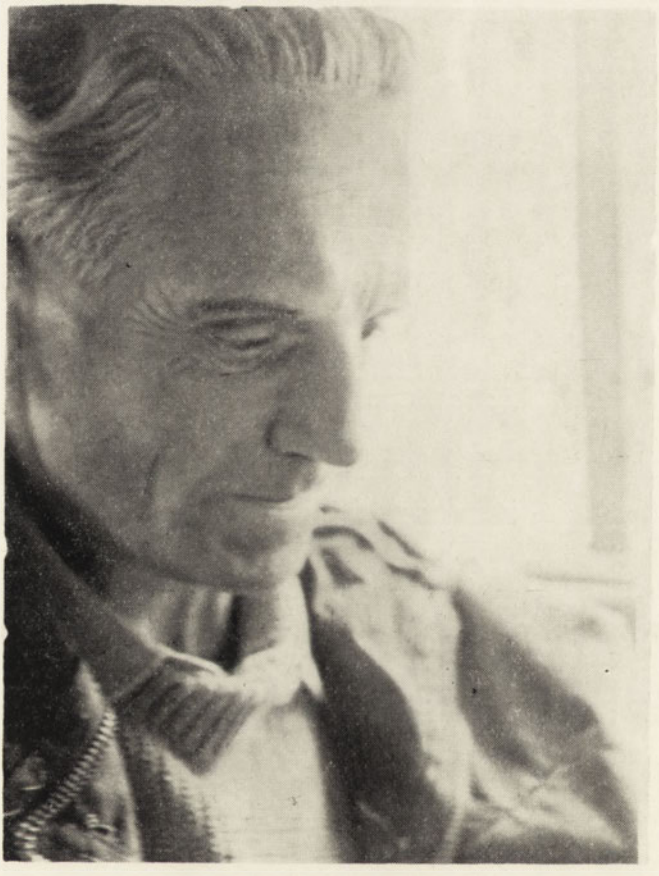

WITOLD BRZESKI

1914—1975

On the 12th of August, 1975, Professor Witold Brzeski, Ph. D., Head of the Department of Biochemistry at the Institute of Plant Biochemistry of the Agricultural University in Warsaw, former Dean of the Faculty of Agriculture, left for ever the ranks of Polish scientific workers.

Witold Brzeski was born in Warsaw in 1914. There he attended secondary school and studied pharmacy at the University. During the war, in 1940, he obtained his M. Sc. degree in pharmacy at the Underground University.

Immediately after the war, Witold Brzeski started to work at the Agricultural University, and to this School he remained faithful for the next 30 years, till the end of his busy life. In the most difficult post-war period, he participated with utmost devotion in the organization of teaching and research work, first as an assistant at the Chair of General Chemistry, then at the Chair of Biochemistry, under the guidance of the late Professor Ignacy Reifer.

At the beginning of his scientific work, Witold Brzeski was interested mainly in the chemical composition of plant products. He obtained his Ph. D. degree in 1957 after having submitted as his dissertation "Studies on the Chemical Composition of Ripe Seeds of *Ricinus pulaviensis*: the Effect of Ferricyanide". In 1956 he worked under Professor E. Broda at the University of Vienna on ^{14}C incorporation into chlorophyll in *Chlorella*. Back in Poland, he shared his experience with radioisotopes by co-editing a collective volume on "Isotopic Methods in Studies on Plant Metabolism" (1960). The results obtained with *Chlorella* he presented in the work: "Incorporation of ^{14}C-labelled Glycine into Chlorophyll *a* and *b*", submitted in 1961 as his thesis for the title of Dozent. At the same time, he started research on regulation of biosynthesis and metabolism of lupin alkaloids.

In 1963 he became the head of the Department of Biochemistry where under his guidance the work centered on the microbiological degradation of quinolysidine alkaloids. A microorganism degrading lupanine: *Pseudomonas lupanini*, has been isolated from soil. Identification of the lupanine

degradation products pointed to the oxidative character of this process and revealed interesting aspects of the induction of lupanine hydroxylase. The results obtained inspired further work on the induction mechanism with this model system.

Parallelly with the research in enzymology, Professor Brzeski was concerned with problems of practical importance in agricultural biochemistry and established bioindices for evaluation of the breeding programme of cultivated plants.

From the very beginning of his work, Witold Brzeski took a very active part in teaching. He prepared several manuals and instructions for laboratory courses. One of these, the "Laboratory Manual of Plant Biochemistry", written together with Prof. Z. Kaniuga, served for many years as the main text-book for students of biochemistry all over Poland.

Professor Witold Brzeski was a member of the Council of the State Publishing House for Agriculture and Forestry, for several years a co-editor of the scientific quarterly *Postępy Biochemii* (Progress in Biochemistry), and an active member of the Polish Biochemical and Biophysical Societies. He was awarded high State distinctions and a golden medal of merit from his own School.

Witold Brzeski died prematurely, in the prime of his scientific career, deeply engaged in all his numerous duties. We have lost in him a devoted research worker, a conscientious teacher of a large group of young biochemists, a teacher highly esteemed by, and popular with, his students. All who came in touch with him appreciated his broad views and understanding of the fundamental problems of biology, his warmth and kindness towards other people.

Maria Toczko

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I. Z. SIEMION and LUCYNA KANIA

FLUORESCENCE OF TYROSINE RESIDUES IN THE BASIC TRYPSIN INHIBITOR FROM BOVINE LUNGS

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Fluorescence of 0.02 - 2% water solutions of basic trypsin inhibitor in the temperature range of 5 - 80°C at pH 2.6 and 7.7 has been investigated and changes of the relative emission at 302.5 and 307.5 nm analysed.

The observed fluorescent effects were ascribed to individual tyrosine residues in the protein molecule.

The temperature-dependent changes of spectra were discussed in terms of possible influence of molecular aggregation in solution at higher protein concentrations.

In a recent investigation on hydrogen-deuterium exchange (Kania *et al.*, 1974) in the basic trypsin inhibitor from bovine lungs at pD 2.6 we observed at 65°C a thermal transconformation of the protein molecule. Simultaneously in n.m.r. spectra no considerable changes appeared up to 75°C; this could suggest that aromatic amino acids are not involved in these conformational changes. Our results are in good agreement with the data obtained by Wütrich (1973) who observed no changes in n.m.r. spectra of this protein below 80°C. Nevertheless, between 40 - 65°C a total exchange of all amide protons to deuterium atoms took place, which was expressed by disappearance of the corresponding peaks in the n.m.r. spectrum. According to Wütrich (1973) this was due to fluctuations of the protein molecule from an average stable conformation. In connection with these results we investigated the fluorescence spectra of the trypsin inhibitor. It is known (Tschernickij, 1972) that this experimental approach can give insight into changes of protein conformation, in regions of aromatic amino acid residues.

MATERIALS AND METHODS

Reagents. Basic trypsin inhibitor from bovine lungs (Kunitz inhibitor) in form of hydrochloride or un-ionized protein was a gift from Zakłady Farmaceutyczne Polfa (Jelenia Góra, Poland). Both samples were homogeneous on zone electrophoresis on cellulose polyacetate (Gelman Inc. Co., Ann Arbor, Mich., U.S.A.).

Fluorescence measurements were performed on a Perkin-Elmer 204 spectrofluorimeter by the stepwise technique at room temperature, the protein concentration being 0.02 - 2%. The pH was checked on an Elpo pH-meter, type N-152 (Wrocław, Poland) and adjusted to the required value by adding a suitable amount of NaOH or HCl solution.

The temperature effects were studied in thermostated 0.5 cm cells. After reaching the appropriate temperature, the samples were equilibrated during 10 min before reading. Readings were made every 2.5 nm and corrected by subtracting the blank spectrum of water.

Fluorescence of the sample is expressed on the figures in the units of emission scale of the apparatus. The sensitivity of the apparatus was adjusted arbitrarily for each series of measurements being compared.

RESULTS AND DISCUSSION

Fluorescence of the pancreatic trypsin inhibitor was investigated by Cowgill (1967), who on iodination observed disappearance of fluorescence after binding of 4 equivalents of iodine. He assumed formation of diiodo-derivatives and concluded that, of the four present tyrosines (Tyr-10, Tyr-21, Tyr-23, and Tyr-35), only two were iodinated, and that these two tyrosines situated on the molecule surface, were responsible for the fluorescence. Quenching of fluorescence of two remaining tyrosines, located in the hydrophobic interior of the molecule, was ascribed by Cowgill (1966) to hydrogen bond formation. In fact, Meloun *et al.* (1968) on nitration of pancreatic trypsin inhibitor found that only Tyr-10 and Tyr-21 were modified. On the other hand, Sherman & Kassel (1968) observed formation of diiodo-derivatives (Tyr-35) and monoiodo-derivatives (Tyr-10 and Tyr-21) during iodination. Their results do not agree with the Cowgill's (1967) interpretation of the origin of the observed fluorescence, as not two but three of the existing tyrosines can be fluorescent. From the investigations on the tertiary structure of the inhibitor molecule (Huber *et al.*, 1970) it is evident that the Tyr-23, which is probably non-fluorescent, is buried in the interior of the protein molecule and additionally shielded by the nearby disulphide bridge.

Formation of monoiodo-derivatives of Tyr-10 and Tyr-21, and diiodo-derivative of Tyr-35 could be explained by the differences in the character of the micro-environment of each residue. Straub (1971) demonstrated on 3-phosphoglyceraldehyde dehydrogenase that iodination of tyrosines located in the apolar regions of the molecule led to diiodo-derivatives, while tyrosines located in the polar micro-environment gave only monoiodo-derivatives. Thus, it may be assumed that in the basic trypsin inhibitor Tyr-10 and Tyr-21 are located in a polar, and Tyr-35 an apolar environment. This supposition is in agreement with the location obtained from X-ray analysis (Huber *et al.*, 1970).

In our fluorescence measurements protein concentration was between 0.02 and 2%. The use of relatively high concentration was necessary for comparison with the results obtained in i.r. investigations of this protein (Kania *et al.*, 1974), where

about 5% protein solutions were used. The fluorescence of 2% solution was low because of strong quenching effects. The fluorescence intensity was the highest with the 0.2% protein solution (Fig. 1) and decreased rapidly with the increase in protein concentration. At low concentrations, the intensity of fluorescence at pH 7.7 did not differ from that at pH 2.6. At both pH values, the maximum of fluorescence was at 300 - 303 nm, and the maximum of the excitation spectrum at 280 nm. For protein concentrations higher than 0.3% the maximum of the excitation spectrum was shifted towards the higher wavelengths, which was probably due to the inner filter effect of the protein solution. The changes of fluorescence spectra with changes in concentrations of protein solution, are illustrated in Fig. 2, where the selected data for pH 2.6 are given.

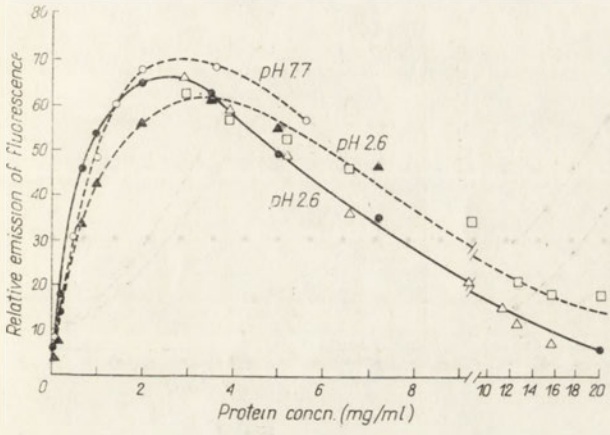

Fig. 1

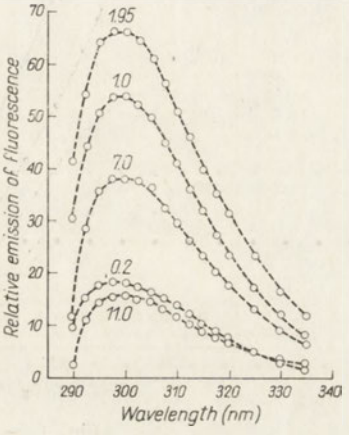

Fig. 2

Fig. 1. Effect of concentration of the basic trypsin inhibitor on the fluorescence (at the maximum of the emission curve), at the indicated pH values. Excitation wavelength: — — —, 285 nm; ——, 280 nm. For the experiments at pH 2.6, the data obtained from two independent dilution series are distinguished by different symbols.

Fig. 2. Effect of concentration of the basic trypsin inhibitor on fluorescence spectra. Excitation wavelength 280 nm, pH 2.6; inhibitor concentration (mg/ml) indicated in the Figure.

In the fluorescence spectrum recorded for 2% protein solution at pH 2.6 and 20°C, excited at 280 nm, two maxima, at 302.5, and 307 - 310 nm were observed (Fig. 3). Raising of temperature to 50°C caused a decrease of emission intensity, but the shape of the curve was retained. A further increase in temperature, up to 70°C, caused flattening of the curve and formation of a broad fluorescence band with a maximum at about 307 nm. The same sample excited at 285 nm showed a considerable increase of fluorescence, but with only one maximum. The increase in temperature caused a decrease in emission intensity but the shape of the fluorescence curve was unchanged.

Fluorescence intensity of 2% protein solutions was distinctly dependent on pH of the medium. In neutral water medium (pH 7.7) fluorescence of the protein excited

at 280 nm reached only half of the value obtained in the acidic medium (Fig. 4). It should be noted that the two samples of lung basic trypsin inhibitor used, i.e. the hydrochloride neutralized to pH 7.7, and the un-ionized form of the protein, showed the same dependence of fluorescence on pH.

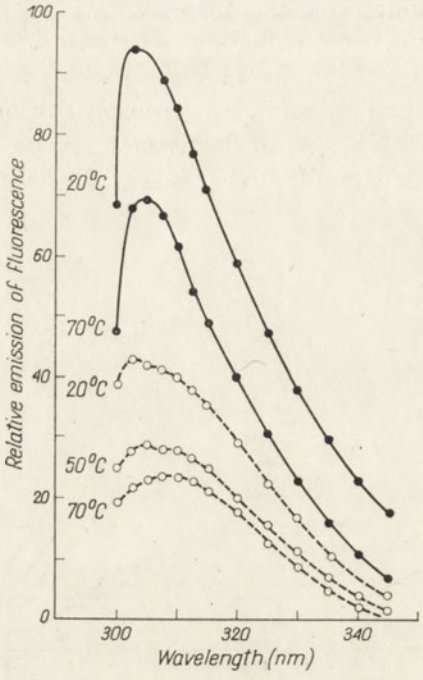

Fig. 3

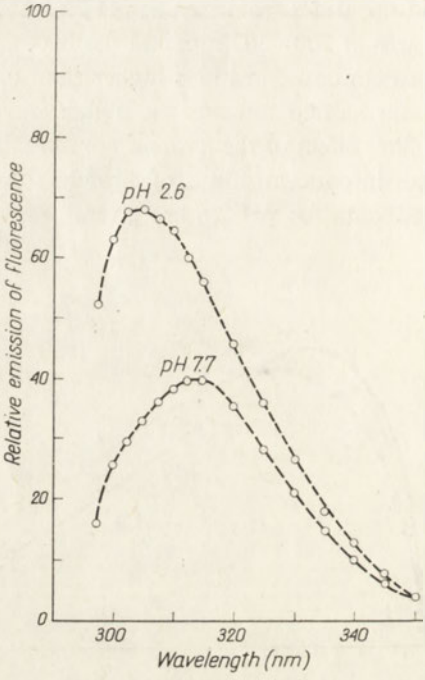

Fig. 4

Fig. 3. Fluorescence spectra of 2% water solution of basic trypsin inhibitor hydrochloride (pH 2.6). Exc. wavelength 285 nm (●) and 280 nm (○) at indicated temperature.

Fig. 4. Fluorescence spectra of 2% basic trypsin inhibitor solution: in 0.2 M-HCl (pH 2.6) and H_2O (pH 7.7). Exc. wavelength 280 nm, at 20°C.

The fluorescence spectrum of the trypsin inhibitor, which was observed after excitation of its 2% solution at 280 nm (Fig. 4) in acidic medium, suggested that the individual tyrosine residues differed somewhat in the position of the maximum in the emission spectrum. This effect, not visible at low protein concentrations, indicates, as it seems, that the different quenching degree of the fluorescence of individual tyrosines is caused by the increase of protein concentration. It seems possible to assume that the maximum of fluorescence at shorter wavelengths can be ascribed to Tyr-10 and Tyr-21, located in the polar micro-environment, and that at longer wavelength, to Tyr-35 located in the apolar region. This assumption is confirmed by the changes in the fluorescence spectrum which are observed in 2% solution after neutralization (Fig. 4).

The strong quenching of fluorescence observed at neutral pH can be evoked by ionization of carboxylic groups located in the vicinity of the tyrosine residues. It is known that phenols can lose their fluorescence during collision with carboxylate

ions. Non-fluorescent tyrosine residues might therefore exist in regions of the protein surface rich in carboxylic groups (Teale, 1960; Rosenheck & Weber, 1964; Cowgill, 1966; 1968). In fact, in the basic pancreatic trypsin inhibitor molecule, Glu-7 is located in the vicinity of Tyr-10, and Glu-49 and Asp-50 near Tyr-21. It should be noted that in the basic inhibitor isolated from bovine lung, Asp-50 is replaced by Asn (Anderer & Hörnle, 1966).

The pH-dependent changes of fluorescence were apparent only at higher protein concentrations which may point to aggregation of protein molecules leading to limitation of conformational freedom in the regions of tyrosine residues.

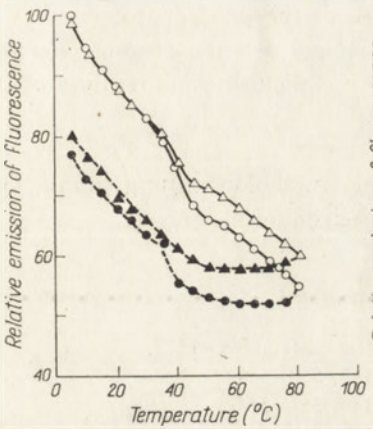

Fig. 5

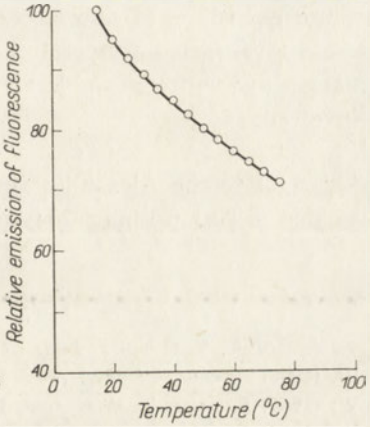

Fig. 6

Fig. 5. Effect of temperature on the emission at 302.5 nm (○, ●) and at 307.5 nm (△, ▲) of 2% solution of basic trypsin inhibitor (pH 2.6): upon heating (solid lines) and recooling (dashed lines).

Fig. 6. Effect of temperature on the emission at 300 nm of 0.2% solution of basic trypsin inhibitor at pH 2.6.

The structure of the trypsin inhibitor molecule is characterized by a strong thermostability. According to Vincent *et al.* (1971) the temperature of thermal transconformation of the protein in an acidic medium is 81°C. Kania *et al.* (1974) observed limited structural changes at about 65°C. In connection with these results we investigated the changes in fluorescence of inhibitor solutions at pH 2.6, in the temperature range of 5 - 80°C, and the excitation wavelength of 280 nm. For the 2% solutions the relative decrease of fluorescence was measured at 302.5 and 307.5 nm (Fig. 5). At 302.5 nm the decrease of fluorescence was linear up to 45°C, the slope being about −0.7% per degree, which is a typical value for the temperature-dependent loss of fluorescence of tyrosine in the absence of conformational changes in protein (Gally & Edelman, 1962; Cowgill, 1968). Above 45°C a distinct change of the slope to a value of −0.3% per degree was observed. At 307.5 nm the changes were quite similar, but the change of the slope appeared at a somewhat higher temperature (50°C). All these changes were not completely reversible. During cooling of the protein solution to 40°C, the low fluorescence value obtained at the highest

investigated temperature, was maintained. Below 40°C an increase of fluorescence was observed; the slope was the same as for the increasing temperature, but the emission was lower. Therefore partial denaturation cannot be excluded.

In 0.2% protein solution the decrease in fluorescence intensity with temperature was nearly linear for the whole investigated temperature range (Fig. 6) and the observed changes were completely reversible on cooling. The slope of the curve was the same as for 2% solution at temperatures over 50°C. It seems that the results obtained for 2% trypsin inhibitor solution reflect the aggregation state of the molecules. In agreement with this assumption it can be concluded that at temperatures over 45 - 50°C the protein aggregates are destroyed.

Temperature quenching of fluorescence of the diluted protein solution (0.2%) over the whole investigated temperature range did not demonstrate any conformational changes in the protein molecule in which tyrosine residues of the protein would be involved.

We are grateful to the Alexander von Humboldt-Stiftung (Bonn, G.F.R.) for making available a Perkin-Elmer 204 spectrofluorimeter.

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FLUORESCENCJA RESZT TYROZYNOWYCH W ZASADOWYM INHIBITORZE TRYPSYNY

Streszczenie

Badano fluorescencję wodnych 0.02 - 2% roztworów zasadowego inhibitora trypsyny, w zakresie temperatur 5 - 80°C, przy pH 2.6 i 7.7, analizując zmiany względnego natężenia fluorescencji przy 302.5 oraz 307.5 nm. Na tej podstawie przypisano obserwowane efekty fluorescencji poszczególnym resztom tyrozynowym w białku. Przedyskutowano możliwość wpływu agregacji cząsteczek białka na jego fluorescencję.

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Z. WASYL

MOLECULAR PROPERTIES OF MULTIPLE FORMS OF ACID PHOSPHATASE FROM HORSE LIVER

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1. Horse liver acid phosphatase was separated into two partially purified fractions differing in molecular weight (enzyme I about 100 000, enzyme II about 25 000).
2. Enzyme I was separated into several subfractions by DEAE-cellulose chromatography and isoelectric focusing.
3. Molecular weight, sedimentation coefficient and effective molecular radii were determined for acid phosphatases I and II by gel filtration and density-gradient centrifugation.

Physico-chemical and molecular properties of acid phosphatase from human prostate are well established (Ostrowski, 1968; Ostrowski & Wasyl, 1969; Ostrowski *et al.*, 1970; Dziembor *et al.*, 1971; Derechin *et al.*, 1971). Chromatographic and electrophoretic heterogeneity of acid phosphatase from bovine and rat liver, as well as some molecular parameters of this enzyme, were investigated by Heinrikson (1969) and Igarashi & Hollander (1968). In the present work, the presence of multiple forms of acid phosphatase from horse liver was demonstrated by gel filtration, column chromatography, isoelectric focusing and density-gradient centrifugation.

MATERIALS AND METHODS

The following reagents, obtained from the indicated sources, were used: *p*-nitrophenylphosphate, disodium salt (Sigma 104) and pepsin (Sigma Chem. Comp., St. Louis, Mo., U.S.A.); α-naphthylphosphate, disodium salt (Goedecke, Freiburg, G.F.R.); bovine serum albumin (B.D.H., Poole, Dorset, England); soybean trypsin inhibitor (Mann Research Lab., N.Y., U.S.A.); human serum albumin (Behringwerke, Marburg, G.F.R.); human γ-globulin (Wytwórnia Surowic i Szczepionek, Warszawa, Poland); $K^{131}I$ (Institute for Nuclear Research, Świerk, Poland);

Sephadex G-200 and Blue Dextran (Pharmacia, Uppsala, Sweden); Ampholine, of pH range 3 - 10 (LKB, Stockholm, Sweden); Fast Blue and DEAE-cellulose (Serva, Heidelberg, G.F.R.). Other reagents were analytical grade products of Polish origin.

Acid phosphatase from human prostate was purified as described by Ostrowski (1968). Bovine serum albumin labelled with ^{131}I was prepared according to Bocci (1969).

Assay of acid phosphatase activity. The enzyme activity was routinely measured at 37°C and pH 5.0 by the rate of liberation of *p*-nitrophenol from 0.02 M-*p*--nitrophenylphosphate in 0.1 M-citrate buffer. The assay mixture contained 0.2 ml of substrate and 0.05 or 0.1 ml of enzyme solution. After incubation for 10 or 60 min, 0.5 M-NaOH was added and the absorbance was measured at 400 nm against an appropriate blank. The enzyme activity was linear with time up to 60 min. The activity is expressed in units, corresponding to micromoles of substrate hydrolysed per minute at 37°C, and the specific activity, as units per 1 mg of protein.

Protein assay. Protein concentration was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard. In the chromatographic and electrophoretic fractions, protein was estimated by measuring absorbance at 280 nm.

Analytical gel filtration on Sephadex G-200. A column of Sephadex G-200 (1.7 × × 47 cm) was equilibrated with 0.05 M-Tris-HCl buffer, pH 7.4, containing 0.1 M--KCl. The preparation of acid phosphatase after ammonium sulphate fractionation (see below) was dialysed for 24 h at 4°C against the equilibration buffer, and 1 ml was applied to the column. The column was calibrated with the following markers: soybean trypsin inhibitor, pepsin, bovine serum albumin, acid phosphatase from human prostate, and human γ-globulin. Samples of each protein (5 mg) and Blue Dextran were dissolved in 1 ml of the equilibrating buffer and layered on top of the column. The elution was carried out with the same buffer at a flow rate of 6 ml/h, and 1.5 ml fractions were collected. The activity of acid phosphatase was estimated in 0.5 ml portions, to which 1.5 ml of substrate solution was added, and the mixture was incubated at 37°C for 60 min.

Sucrose-density-gradient centrifugation. This was carried out according to Martin & Ames (1961) in a Janetzki VAC-60 preparative ultracentrifuge using a swinging bucket rotor (3 × 5 ml). A linear gradient of sucrose from 5 to 20% (w/v) in 0.05 M--Tris-HCl buffer, pH 7.4, or in the same buffer containing 0.15 M-NaCl, was used. A 0.2 ml sample of acid phosphatase containing 10 µl of a solution of bovine serum albumin labelled with ^{131}I, was layered on top of the gradient (4.6 ml) and centrifuged at about 5°C for 16 h at 38 000 rev./min. Fractions of 4 drops each were collected and analysed for acid phosphatase activity and concentration of ^{131}I--labelled albumin by measuring the count rate in a well-type scintillation counter (USB-2, Polon, Warszawa, Poland).

Isoelectric focusing. Isoelectric fractionation of the acid phosphatase (about 4 units) was performed according to the procedure of Vesterberg & Svensson (1966) with the use of an LKB apparatus (LKB Instruments, Bromma 1, Sweden), (110 ml

column, 1% Ampholine of pH range 3 - 10). The separation was carried out for 48 h at 5°C and 300 V. Fractions of 1.8 ml were collected and pH was measured at 5°C. Protein was assayed at 280 nm and activity of acid phosphatase by the standard method.

Chromatography on DEAE-cellulose. The separation was carried out on a column (1 × 15 cm) of DEAE-cellulose which had been equilibrated with 0.05 M-Tris-HCl buffer, pH 7.4. A sample of acid phosphatase preparation, 8 ml, dialysed previously against the same buffer, was applied to the column and eluted with a linear NaCl concentration gradient (0 - 0.3 M) in the same buffer. Fractions of 2.5 ml were collected at a rate of 10 ml/h.

Initial purification of horse liver acid phosphatase. The purification procedure was carried out at 4°C, and is summarized in Table 1. Fresh horse liver was homogenized with 0.9% NaCl solution in a Waring Blendor for 3 min. The homogenate was centrifuged at 4800 *g* for 20 min (Janetzki K-60 refrigerated centrifuge). About 90% of the activity was recovered in the supernatant fraction. The supernatant was adjusted to pH 5.0 with 1 M-acetic acid. After 12 h, the precipitate was centrifuged off and to the supernatant, solid ammonium sulphate was added gradually, with slow stirring, to 0.5 saturation. After 60 min of stirring, the precipitate was centrifuged off, and ammonium sulphate was added to 0.8 saturation. The mixture was kept overnight with slow stirring; the precipitate collected by centrifugation was dissolved in 0.05 M-Tris-HCl buffer, pH 7.4, dialysed for 48 h against the same buffer, and any precipitate formed removed by centrifugation at 20 000 *g*.

Table 1

Summary of purification procedure of acid phosphatase from horse liver

For purification, 300 g of liver was used.

Fraction	Total volume (ml)	Total protein (mg)	Activity		Purification factor	Recovery (%)
			total (units)	specific (units/mg)		
Homogenate	1240	115 000	1358	0.01	1.0	(100)
pH 5 supernatant	1250	27 034	446	0.016	1.6	33
Ppt. at 0.5 - 0.8 $(NH_4)_2SO_4$ sat.	100	4 320	342	0.08	8.0	25.1
Sephadex G-200						
Peak I	26	190	48.8	0.24	24.0	3.6
Peak II	27.5	203.5	26.1	0.135	13.5	1.9

The dialysed enzyme solution, 50 ml, containing about 170 units, was applied to a column (6 × 110 cm) of Sephadex G-200 equilibrated with 0.05 M-Tris-HCl buffer, pH 7.4, containing 0.1 M-KCl. The elution was carried out with the same buffer and fractions of 6 ml were collected at a flow rate of 24 ml/h. Two peaks of enzyme activity (enzyme I and II) were obtained. Fractions containing more than 0.1 unit of enzyme activity per 1 ml, were pooled. The acid phosphatases I and II were precipi-

tated with ammonium sulphate between 0.5 and 0.8 saturation; the precipitate was dissolved in 0.05 M-Tris-HCl buffer, pH 7.4, containing 0.1 M-KCl, and dialysed against the same buffer.

RESULTS

Heterogeneity of acid phosphatase with respect to molecular weight. The preparation of horse liver phosphatase obtained by ammonium sulphate fractionation, separated on Sephadex gel filtration into two active peaks (Fig. 1). Both fractions had the same pH optimum at 4.85 (Fig. 2). The molecular weight estimated by gel filtration was about 100 000 for enzyme I and 23 000 for enzyme II (see Fig. 6). On sucrose-gradient centrifugation both enzymes gave single symmetrical peaks (Fig. 3a,b), acid phosphatase I sedimenting faster than enzyme II.

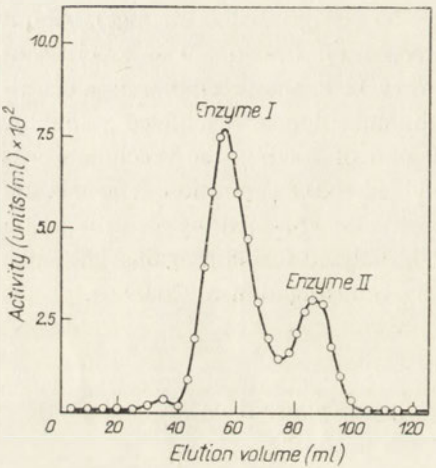

Fig. 1

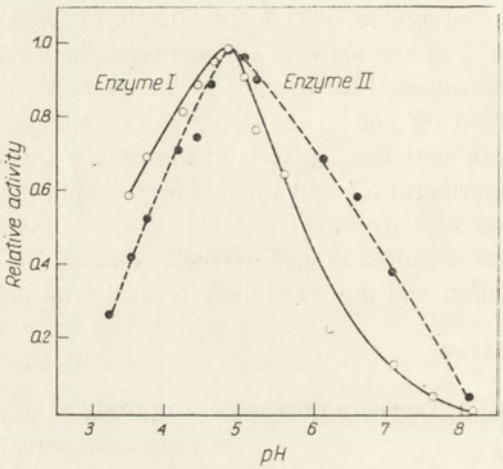

Fig. 2

Fig. 1. Sephadex G-200 gel filtration of acid phosphatase from horse liver. The column was equilibrated with 0.1 M-KCl - 0.05 M-Tris-HCl buffer, pH 7.4, and eluted with the same buffer.

Fig. 2. pH activity curves of acid phosphatase I and II from horse liver. The buffers used were: acetate, pH 3.5 - 5.6; Tris-maleate, pH 6.2 - 8.1. Enzyme I or II, 0.05 ml, was incubated for 10 min at 37°C with 0.5 ml of the appropriate buffer containing 20 mM-*p*-nitrophenylphosphate.

Electrophoretic heterogeneity. The extract of acid phosphatase from horse liver, after ammonium sulphate fractionation, gave on isoelectric focusing several peaks of enzymic activity in the pH range of 3 - 10 (Fig. 4a). About 14% of the eluted activity was recovered in peaks with isoelectric points over the pH range 3.6 - 4.8, about 36% at pH 5.3, and 27% and 23% of the activity at pH 6.2 and 6.8, respectively.

The acid phosphatase I gave on isoelectric focusing several active peaks (Fig. 4b), with isoelectric points similar to those of the ammonium sulphate fraction. On the other hand, acid phosphatase II gave only two peaks (Fig. 4c), about 87% of the activity being recovered at pH 6.2, and about 11% at pH 5.2.

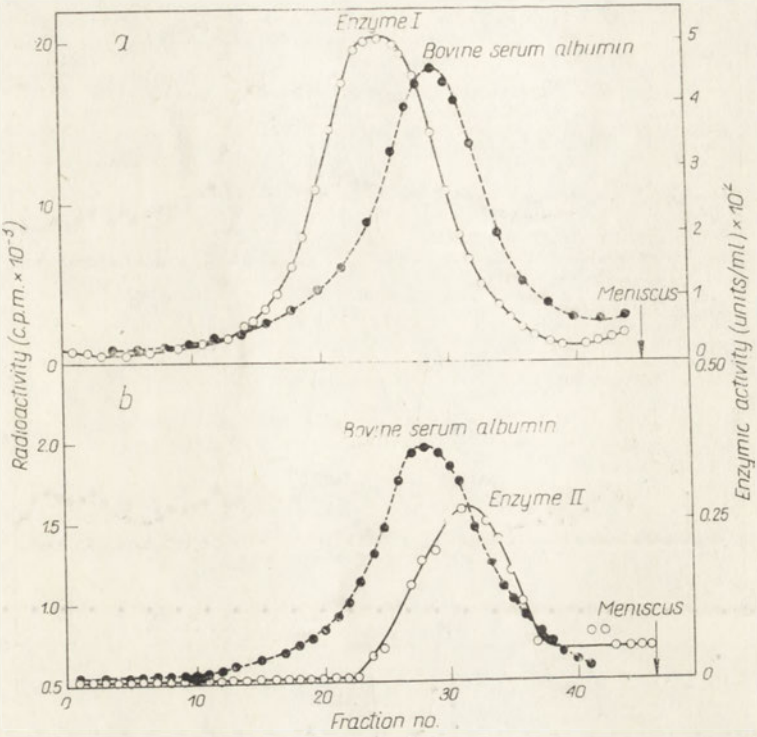

Fig. 3. Sucrose-density-gradient centrifugation of *a*, acid phosphatase I from horse liver in 0.05 M-Tris-HCl buffer, pH 7.4; and *b*, acid phosphatase II in the same buffer containing 0.15 M-NaCl. ○, Enzymic activity; ●, radioactivity of ^{131}I-labelled bovine serum albumin used as a marker.

Table 2

Some physico-chemical constants of acid phosphatases I and II from horse liver

	Acid phosphatase	
	I	II
Stokes radius (Å)	44.0	20.0
Diffusion coefficient, $D_{20,w} \times 10^7$	4.86	10.7
Sedimentation coefficient, $s^{0.725}_{20,w} \times 10^{13}$	5.57	3.14
Molecular weight		
determined by gel filtration	100 000	23 000
determined from Svedberg equation	102 000	25 000
Frictional ratio, f/f_0	1.42	1.03
Isoelectric point	4.1 (7%)	5.1 (11%)
	5.06 (36%)	
	6.2 (27%)	6.2 (87%)
	6.6 (23%)	

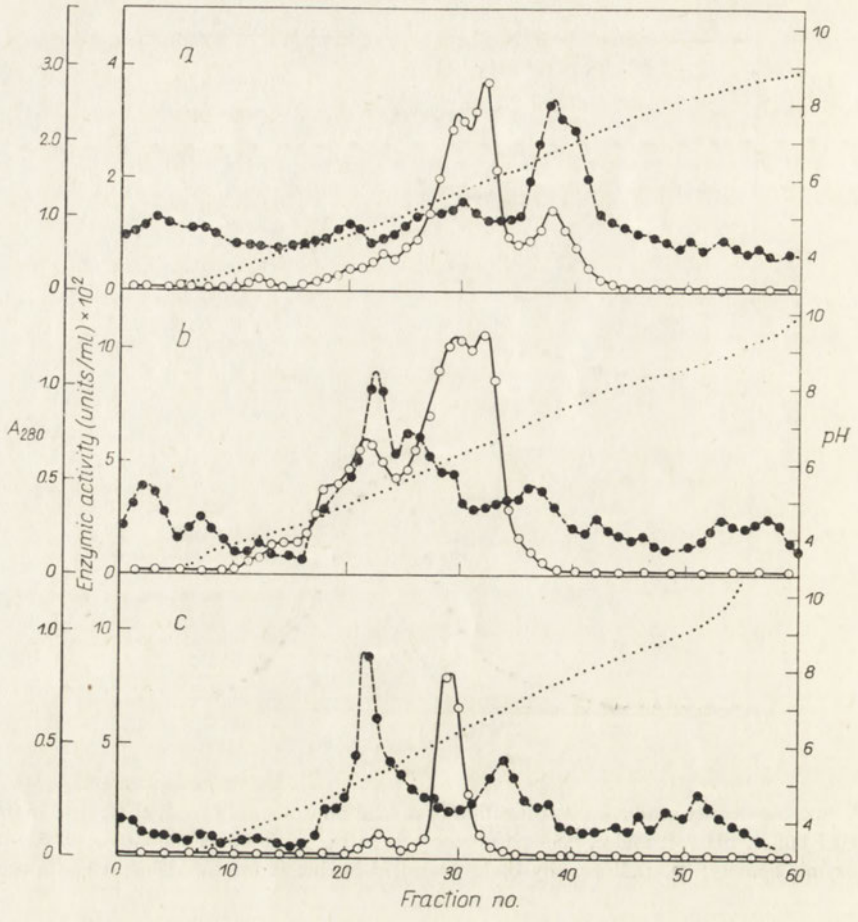

Fig. 4. Isoelectric fractionation of acid phosphatase from horse liver: *a*, fraction obtained at 0.5 - 0.8 ammonium sulphate saturation; *b*, acid phosphatase I; *c*, acid phosphatase II. ○, enzymic activity; ●, protein; ···, pH gradient.

Chromatographic heterogeneity. On DEAE-cellulose chromatography, acid phosphatase I separated into two active peaks (enzymes *A* and *B*, Fig. 5a); about 72% of the activity was present in peak *A*, and 28% in peak *B*. Under the same conditions, acid phosphatase II gave a single active peak (Fig. 5b) which emerged at the same NaCl concentration as peak *A* of acid phosphatase I.

Molecular parameters. The molecular weight and Stokes radius of acid phosphatases I and II were estimated by column gel filtration on Sephadex G-200. The molecular weight obtained by the method of Andrews (1965) for the two enzymes was, respectively, 100 000 and 23 000 (Fig. 6). From the plot based on the equation of Laurent & Killander (1964), the molecular radius of acid phosphatases I and II was determined to be, respectively, 44 and 20 Å (Fig. 7).

The obtained and calculated molecular parameters of the two acid phosphatases from horse liver are summarized in Table 2. The diffusion coefficient, $D_{20,w}$, of the enzyme I and II, calculated from the Stokes-Einstein equation, was 4.86×10^{-7} cm^2sec^{-1} and 10.7×10^{-7} cm^2sec^{-1}, respectively. The sedimentation coefficient was determined according to Martin & Ames (1961), assuming partial specific volume 0.725 cm^3/g, as 5.57×10^{-13} sec and 3.13×10^{-13} sec. The molecular weight of acid phosphatase I and II calculated from gel filtration and ultracentrifugation data according to the classical Svedberg equation, was 102 000 and 25 000, respectively, which is in good agreement with the values obtained by gel filtration.

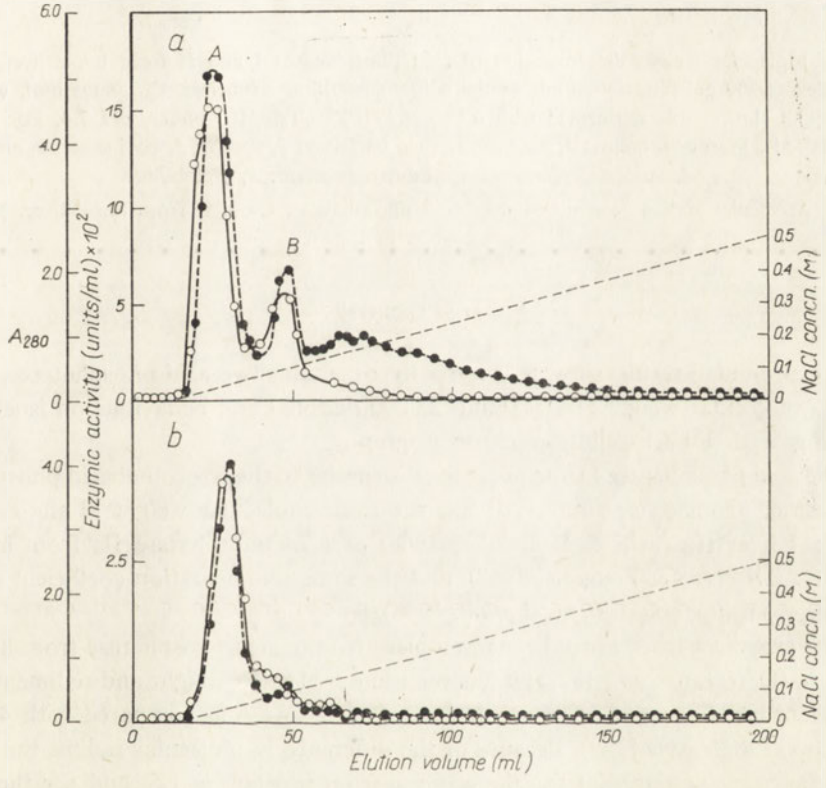

Fig. 5. DEAE-cellulose chromatography of *a*, acid phosphatase I, and *b*, acid phosphatase II from horse liver. The column was equilibrated with 0.05 M-Tris-HCl buffer, pH 7.4, and eluted with increasing concentration of NaCl in Tris-HCl buffer. ○, Enzymic activity; ●, protein; — —, NaCl concentration.

From the effective molecular radius, molecular weight and partial specific volume, it is possible to calculate the frictional ratio f/f_0 (Siegel & Monty, 1966). This ratio for acid phosphatase I and II was found to be 1.42 and 1.03, respectively.

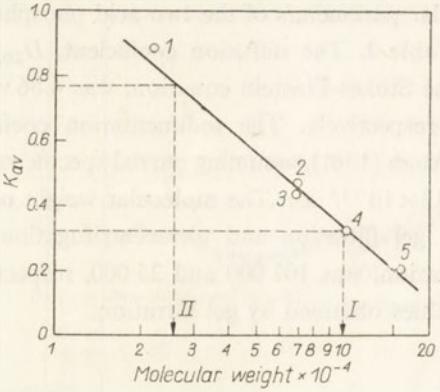

Fig. 6

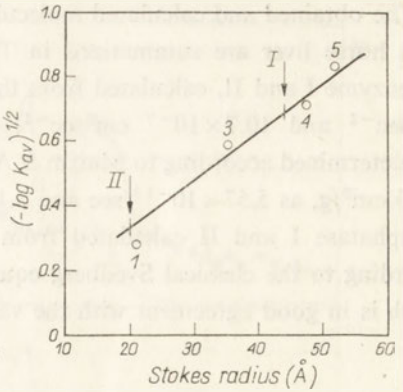

Fig. 7

Fig. 6. Molecular weight determination of acid phosphatases I and II from horse liver, using Sephadex G-200 gel filtration. Plots of the column partition coefficient K_{av} *versus* mol. wt. are given. The elution was performed with 0.1 M-KCl - 0.05 M-Tris-HCl buffer, pH 7.4. For details see Methods. Marker proteins: *1*, Soybean trypsin inhibitor; *2*, pepsin; *3*, bovine serum albumin; *4*, acid phosphatase from human prostate; *5*, γ-globulin.

Fig. 7. Molecular radius determination of acid phosphatases I and II from horse liver. Marker proteins as in Fig. 6.

DISCUSSION

The presented results show that horse liver contains two acid phosphatases, differing in molecular weight (100 000 and 25 000 daltons) and behaviour on isoelectric focusing and DEAE-cellulose chromatography.

The acid phosphatase I from horse liver is similar to the lysosomal acid phosphatases isolated from other sources. It has the same molecular weight as the enzyme from rat liver (Igarashi & Hollander, 1968) or acid phosphatase III from human placenta (Di Petro & Zengerle, 1967), and the same sedimentation coefficient as the enzyme from hog spleen (Chersi *et al.*, 1966).

The enzyme I from horse liver resembles also the acid phosphatase from human prostate (Derechin *et al.*, 1971); it has the same molecular weight and sedimentation coefficient but a somewhat smaller molecular radius, 44 Å as compared with 47.1 Å (Ostrowski & Wasyl, 1969). Because of the difference in molecular radius, the value of the asymmetry constant for the enzyme from prostate is 1.5, and for the liver enzyme, 1.42.

The heterogeneous pattern observed on electrofocusing of the horse liver enzyme resembles that observed by Ostrowski *et al.* (1970) for the prostate acid phosphatase, which separated into a few fractions with isoelectric points ranging from 4.0 to 5.2. On removal of neuraminic acid, the molecular radius of the enzyme from prostate decreased to 45 Å, and on electrofocusing the enzyme gave a single symmetrical peak at pH 6 (Dziembor *et al.*, 1971). A fraction with the same isoelectric point was present also in horse liver phosphatase I, and formed the main fraction of phosphatase II.

It was also found in our laboratory (Wasyl, 1975) that horse leucocytes contain two species of acid phosphatase which resemble closely acid phosphatases I and II from horse liver with respect to molecular weight, effective radius and sedimentation coefficient.

The author is much indebted to Dr. A. Koj for continued interest in this work and stimulating discussion.

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WŁASNOŚCI MOLEKULARNE RÓŻNORODNYCH FORM KWAŚNEJ FOSFATAZY Z WĄTROBY KONIA

Streszczenie

1. Wątroba konia zawiera dwie kwaśne fosfatazy różniące się ciężarem cząsteczkowym (enzym I około 100 000 daltonów, enzym II około 25 000 daltonów).

2. Podczas chromatografii na DEAE-celulozie i podczas izoelektroforezy ogniskującej enzym I ulega rozdzieleniu na kilka podfrakcji.

3. Ciężar cząsteczkowy, efektywny promień oraz współczynnik sedymentacji obu form kwaśnej fosfatazy wyznaczono przy pomocy filtracji na żelu oraz wirowania w gradiencie sacharozy.

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WIESŁAWA ŁYSIAK, MARIA PIEŃKOWSKA-VOGEL, A. SZUTOWICZ and S. ANGIELSKI

EFFECT OF BICARBONATE BUFFER ON THE ACTIVITY OF CYTOPLASMIC AND MITOCHONDRIAL ALANINE AMINOTRANSFERASE*

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1. Bicarbonate stimulates the activity of rat brain cytoplasmic and mitochondrial alanine aminotransferase (EC 2.6.1.2), probably due to the enhanced affinity for its substrates.

2. Under the same conditions, the activity of crystalline aspartate aminotransferase was inhibited.

3. The role of bicarbonate buffer in regulation of alanine aminotransferase activity and synthesis of alanine are discussed.

The effect of bicarbonate on the activity of various enzymes has been extensively studied. Bicarbonate was found to inhibit succinate dehydrogenase (EC 1.3.99.1; Zeylemaker *et al.*, 1970), glucose-6-phosphate dehydrogenase (EC 1.1.1.49; Horne *et al.*, 1970), and mitochondrial and cytoplasmic aconitate hydratase (EC 3.2.1.3; Angielski & Stępiński, 1971). On the other hand, glucose dehydrogenase (EC 1.1.1.47) is stimulated by bicarbonate (Horne & Nordlie, 1971).

Saier & Jenkins (1967b) reported on the enhancement of alanine aminotransferase (EC 2.6.1.2) from rat liver by formate, a monocarboxylic ion similar to HCO_3^-.

The aim of the present work was to study the effect of bicarbonate buffer on the activity of cytoplasmic and mitochondrial alanine aminotransferase[1] and compare it with the effect on crystalline aspartate aminotransferase (EC 2.6.1.1).

MATERIALS AND METHODS

Reagents. L-Amino acids were from Reanal (Budapest, Hungary), 2-oxoglutarate (monopotassium salt), Triton X-100 and lactate dehydrogenase (EC 1.1.1.27) from Sigma Chem. Co. (St. Louis, Mo., U.S.A.), glutamate dehydrogenase (EC 1.4.1.3)

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[1] Abbreviations used: AlAT, alanine aminotransferase; AspAT, aspartate aminotransferase.

and NADH from Boehringer und Soehne GmbH (Mannheim, G.F.R.), malate dehydrogenase (EC 1.1.1.37) and aspartate aminotransferase (EC 2.6.1.1) of hog heart from Koch-Light Lab. Ltd (Colnbrook, Bucks., England). Ficoll, a product of Pharmacia (Uppsala, Sweden), was dialysed and freeze-dried before use. Other reagents were from P.O.Ch. (Gliwice, Poland). $KHCO_3$ was recrystallized under CO_2 and stored in a desiccator.

Preparations of alanine aminotransferase. The 90-fold purified cytoplasmic enzyme was obtained from rat brain after Saier & Jenkins (1967a) as described by Łysiak *et al.* (1974). Specific activity of the preparation obtained was 0.68 μmole of pyruvate formed/mg protein/min.

Crude mitochondrial enzyme was obtained both from rat brain mitochondria isolated according to Clark & Nicklas (1970) and from rat liver mitochondria (Hogeboom, 1957). Since mitochondrial AlAT proved to be a latent enzyme, the isolated mitochondria were treated with Triton X-100 added to a final concentration of 0.5%. Mitochondria were used for experiments not later than 30 min after their isolation. On a 5 min treatment with Triton X-100, AlAT activity was increased in brain mitochondria about sevenfold, and in liver mitochondria, by a factor of 1.5. It seems that the difference in the response to Triton could be in part due to the differences in permeability of mitochondrial membranes from both tissues. The effect of contamination of the brain mitochondrial fraction with synaptosomes should be also taken into account.

Determination of alanine aminotransferase activity. The assay was carried out in glass tubes (9 × 100 mm) at 37°C. The incubation mixture in a final volume of 0.5 ml contained: 3 mM-2-oxoglutarate; 10 or 20 mM-alanine and one of the following buffers: (a) 50 mM-Tris-HCl buffer; (b) 50 mM-potassium phosphate buffer; (c) 50 mM-sodium pyrophosphate buffer, or (d) bicarbonate buffer, in which pH was adjusted to the required value by changing $KHCO_3$ concentration at a constant CO_2 pressure, or CO_2 pressure at constant $KHCO_3$ concentration. The required osmolarity was obtained by adding KCl. During preincubation the samples were saturated with a mixture of oxygen and carbon dioxide of a known pressure, which was maintained throughout the incubation period. pH of the complete reaction mixture was checked in parallel samples, using a PHM 27 pH-meter with an E 5021 capillary electrode (Radiometer, Copenhagen, Denmark).

Following 5 min preincubation, the reaction was started by adding AlAT: 5.7 - 8.1 μg of the cytoplasmic protein, and 0.6 - 0.8 mg or 0.08 - 0.12 mg protein of the brain and liver mitochondria, respectively. The reaction was stopped after 10 min by adding 50 μl of 30% $HClO_4$. The control sample was deproteinized at zero time. The samples were centrifuged for 10 min at 3000 rev./min, and the clear supernatant neutralized with Na_3PO_4. The rate of the reaction was determined by measuring the amount of pyruvate formed in a final volume of 1.5 ml (Rosenberg & Rush, 1966).

Under the applied conditions, the reaction rate was linear with protein concentration and time of incubation.

Determination of aspartate aminotransferase activity. The assay was carried out at 37°C. The incubation mixture in a final volume of 0.5 ml contained: 30 mM-aspartate, 0.3 mM-2-oxoglutarate, 0.1 mM-NADH, 25 μg of malate dehydrogenase, and 20 - 60 ng of AspAT preparation. Following 5 min preincubation, the reaction was started by adding AspAT, and stopped after 7 min by adding 1.1 ml of cold 40 mM-NaOH. Changes in absorption were followed at 340 nm. In the control sample, the reaction was stopped at zero time. The buffers used were 50 mM-Tris-HCl, 50 mM-potassium phosphate, or bicarbonate prepared as described above. The reaction was linear with the amount of added protein and the reaction time.

Protein was assayed spectrophotometrically at 260 - 280 nm or by the biuret method (Layne, 1957) using bovine serum albumin, fraction V, as a standard.

RESULTS

Effect of buffer on alanine aminotransferase activity

Cytoplasmic enzyme. AlAT isolated from rat brain was more susceptible to changes in hydrogen ion concentration when assayed in bicarbonate buffer than in Tris-HCl (Fig. 1A). In the latter buffer, the increment in the reaction rate when passing from pH 6.8 to 7.8 was about 30%, whereas in bicarbonate buffer about 70%. At pH values exceeding 7.3 and a CO_2 pressure of 53 mm Hg, AlAT activity in bicarbonate buffer was higher than in Tris-HCl. At this only CO_2 pressure, at optimum pH, the effect of bicarbonate buffer on AlAT activity was significant ($0.001 < p < 0.005$). In phosphate buffer, the enzyme activity was less than a half that in Tris-HCl.

Over the studied range of CO_2 pressure, pH optimum was practically the same in bicarbonate as in Tris-HCl and phosphate buffers.

When pH was adjusted by changing CO_2 pressure (Fig. 1B), the AlAT activity in 20 mM-bicarbonate buffer was similar to that in Tris-HCl, whereas in 40 mM-bicarbonate it was by about 10% higher. This increase, although slight, was however significant ($0.02 < p < 0.05$). For a given pH, the increase in bicarbonate concentration or CO_2 pressure was associated with an increase in AlAT activity. Also at the optimum pH range the activity of AlAT was the least susceptible to the changes in HCO_3^-/CO_2 ratio.

At constant pH value of 7.40 ± 0.03, an increase in concentration of bicarbonate buffer resulted in an increase in the activity of AlAT, both toward pyruvate (Fig. 2A) and 2-oxoglutarate (Fig. 2B) as substrates. The data presented seem to suggest a possible substrate inhibition at pyruvate concentrations exceeding 1 mM.

Mitochondrial enzyme. The activity of AlAT from brain and liver mitochondria (Fig. 3A,B) was affected by changes in CO_2 pressure in bicarbonate buffer to a lesser extent than the cytoplasmic enzyme. The increase in CO_2 pressure from 17 to 35 mm Hg had practically no effect, whereas at 70 mm Hg the activity was increased only by about 10%. Irrespective of CO_2 pressure, the activity of brain mitochondrial AlAT at the pH optimum was by 15% lower in bicarbonate buffer than in Tris-HCl, and that of liver mitochondrial enzyme, by about 40%.

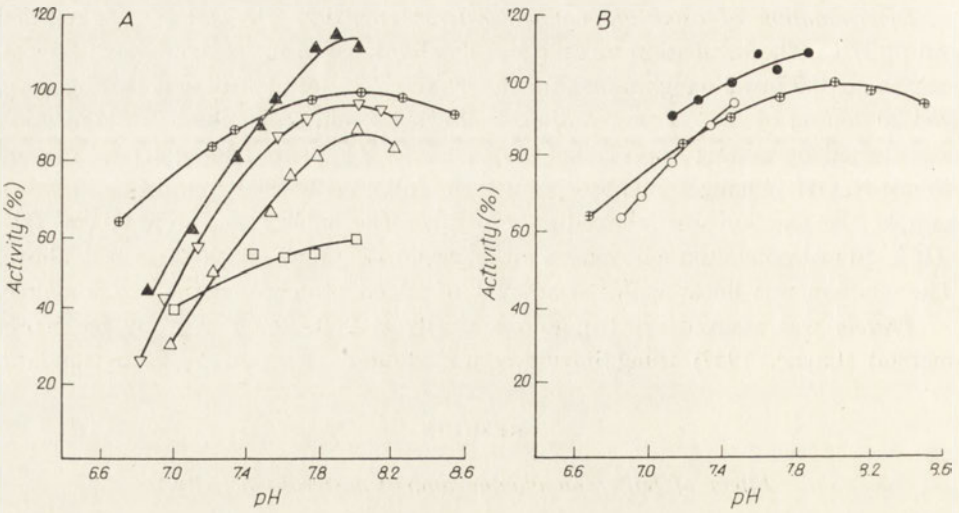

Fig. 1. Effect of the type of buffer on the activity of rat brain cytoplasmic alanine aminotransferase: *A*, at varying concentration of HCO_3^-; and *B*, at varying pressure of CO_2. Concentration of alanine 10 mM, of 2-oxoglutarate 3 mM. The activity is expressed in relation to that obtained in Tris-HCl buffer, pH 8.0, taken as 100. The average specific activity of AlAT was 0.57 μmoles of pyruvate formed/mg protein/min. The results are mean values from 4 - 8 experiments, S. E. = ±1.5%. ⊕, 50 mM-Tris-HCl buffer; □, 50 mM-phosphate buffer. In *A*: bicarbonate buffer at CO_2 pressure of: △, 25 mm Hg; ▽, 35 mm Hg; ▲, 53 mm Hg, with the $KHCO_3$ and KCl concentrations varying, respectively, from 5 to 100 mM and from 95 to 0 mM. In *B*, bicarbonate buffer contained either ○, 20 mM-$KHCO_3$ and 80 mM-KCl, or ●, 40 mM-$KHCO_3$ and 60 mM-KCl, with CO_2 pressure ranging from 17 to 164 mm Hg.

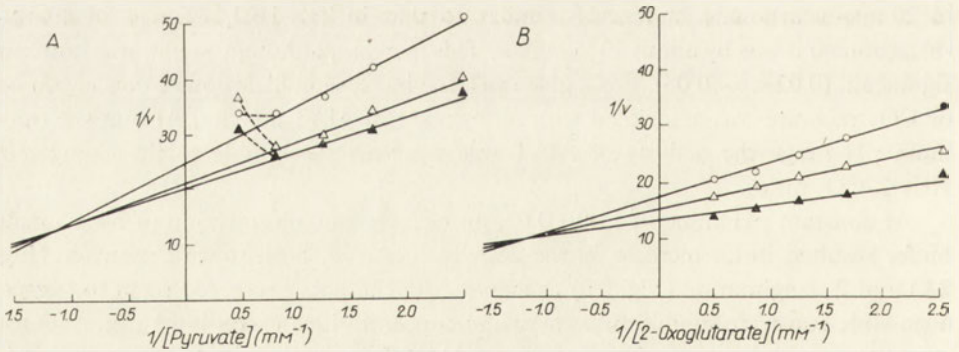

Fig. 2. The activity of cytoplasmic alanine aminotransferase in bicarbonate buffer in the presence of varying concentrations of *A*, pyruvate, and *B*, 2-oxoglutarate. The incubation mixture contained for *A*: 10 mM-L-glutamate and 10.5 μg of protein/sample; for *B*: 10 mM-alanine and 8.1 μg protein/sample, and: ○, 9 mM-$KHCO_3$, 91 mM-KCl (CO_2 pressure 17.7 mm Hg); △, 35 mM-$KHCO_3$, 65 mM-KCl (CO_2 pressure 66 mm Hg); ▲, 60 mM-$KHCO_3$, 40 mM-KCl (CO_2 pressure 114 mm Hg). pH of the incubation mixture was 7.40±0.03. The reaction rate was expressed as μmoles of *A*, 2-oxoglutarate or *B*, pyruvate formed per sample.

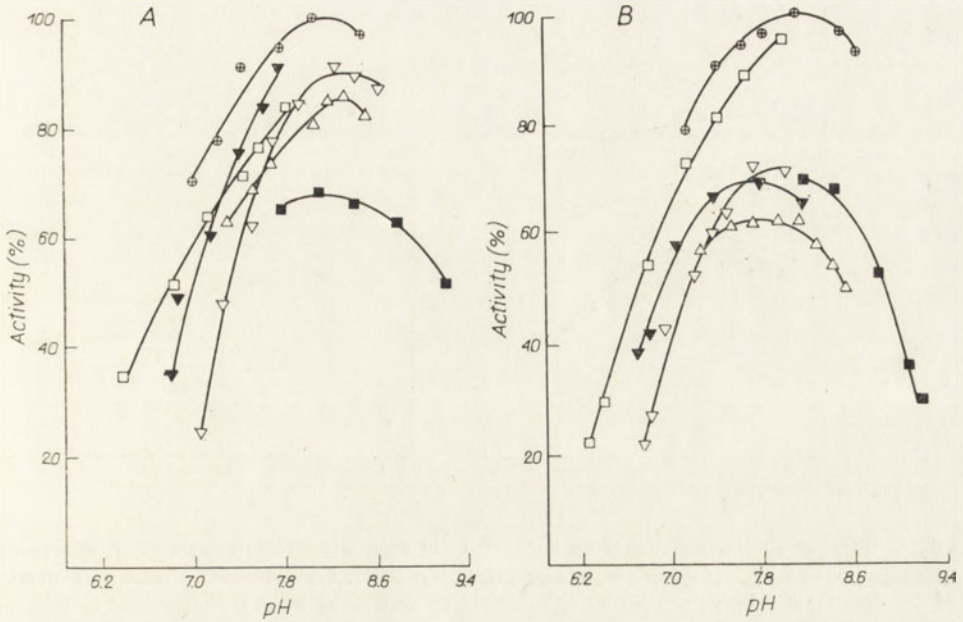

Fig. 3. Effect of the type of buffer on the activity of alanine aminotransferase from mitochondria of *A*, rat brain and *B*, rat liver. The incubation mixture in a final volume of 0.5 ml contained: 10 mM-alanine, 3 mM-2-oxoglutarate, 1 mM-arsenite and alternatively: ⊕, 50 mM-Tris-HCl buffer; □, 50 mM-potassium phosphate buffer; ■, 50 mM-sodium pyrophosphate buffer; or bicarbonate buffer at CO_2 pressure of: △, 17 mm Hg; ▽, 35 mm Hg; or ▼, 70 mm Hg. Other conditions as in Fig.1. The average specific activity of AlAT in Tris buffer, pH 8.0, taken as 100 was in experiments *A* and *B*, respectively, 9.9 and 48 nmoles of pyruvate formed/mg protein/min. The results are mean values from four experiments, S. E. = ±2%.

The mitochondrial enzyme was inhibited in phosphate buffer only by 10 - 15% as compared with 40 - 50% inhibition of the cytoplasmic enzyme. The lowest activity of mitochondrial AlAT was observed in pyrophosphate buffer.

Effect of buffer on the activity of aspartate aminotransferase

Over the studied pH range (Fig. 4A), the activity of AspAT was the highest in Tris-HCl buffer; it was lower by 15% in phosphate buffer, and still lower in bicarbonate buffer. It should be noted that both an increase in CO_2 pressure from 23 to 51 mm Hg at a constant concentration of HCO_3^-, and the increase in bicarbonate concentration from 20 to 40 mM, decreased AspAT activity, respectively, by 20 and 10%.

The activity of AspAT could be affected not only by bicarbonate but also by chloride ion (Cheng *et al.*, 1971). Under the experimental conditions applied, concentration of chloride ranged, depending on the pH value, from 45 to 10 mM in Tris-HCl buffer and from 95 to 0 mM in bicarbonate buffer. However, at the lowest

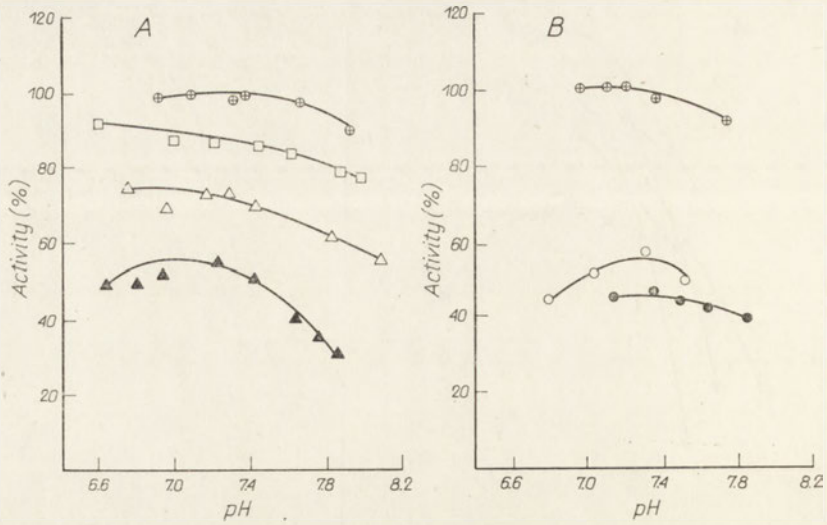

Fig. 4. Effect of the type of buffer on the activity of aspartate aminotransferase: *A*, at varying concentrations of HCO_3^-; and *B*, at varying pressure of CO_2. Concentration of aspartate 30 mM, of 2-oxoglutarate 0.3 mM; protein 40 ng/sample. The activity of AspAT is expressed in relation to that in Tris-HCl buffer, pH 7.2, taken as 100. The average specific activity of AspAT was 198 μmoles of oxaloacetate formed /mg protein/min. The results are mean values from four experiments. ⊕, 50 mM-Tris-HCl buffer; □, 50 mM-potassium phosphate buffer; and in *A*, bicarbonate buffer at CO_2 pressure of: △, 23 mm Hg and ▲, 51 mm Hg; in *B*, bicarbonate buffer containing: ○, 20 mM-$KHCO_3$ and 80 mM-KCl, and ●, 40 mM-$KHCO_3$ and 60 mM-KCl, with CO_2 pressure ranging from 17 to 135 mm Hg.

Cl^- concentration, at pH values exceeding 7.8, the activity of AspAT in bicarbonate buffer was lower than in Tris-HCl by 30 - 60%, depending on CO_2 pressure (Fig. 4A). The activity of AspAT was also decreased, despite lowering of Cl^- level from 80 to 60 mM, when HCO_3^- concentration was raised from 20 to 40 mM (Fig. 4B). Therefore it may be concluded that inhibition of AspAT activity was due solely to the influence of bicarbonate buffer.

K_m *values of alanine aminotransferase*

As it may be seen from Table 1, the kind of buffer used in the incubation mixture affected the affinity of the enzyme for its substrates. With the enzyme from rat brain cytoplasm, the K_m value for alanine was one-third of that in Tris buffer, and with the enzyme from rat liver mitochondria this value in bicarbonate buffer was a half for alanine and one-third for 2-oxoglutarate as compared with the corresponding values obtained in pyrophosphate buffer. The latter values are in agreement with those of Swick *et al.* (1965). In 40 mM-bicarbonate buffer, the K_m values for the enzyme from liver mitochondria were the same as for the enzyme from brain mitochondria. Both, for the cytoplasmic enzyme from rat brain and the mitochondrial enzyme from rat liver, the differences between K_m values determined in pyrophosphate buffer and in bicarbonate buffer, were statistically significant.

Table 1

K_m values for substrates of alanine aminotransferase

The K_m values were determined after Florini & Vestling (1957) in the bicarbonate buffer containing 60 mM-$KHCO_3$ and 40 mM-KCl at CO_2 pressure of 25 mm Hg (pH 7.90±0.05), or 40 mM-$KHCO_3$ and 60 mM-KCl at CO_2 pressure of 23 mm Hg (pH 7.83±0.05), and in 50 mM-sodium pyrophosphate buffer (pH 7.85±0.05). The reaction rate was determined by measuring the amount of pyruvate (Rosenberg & Rush, 1966) or 2-oxoglutarate (Bergmeyer & Bernt, 1965) formed. Concentration of amino acids ranged from 6 to 40 mM and that of 2-oxo acids from 0.2 to 4 mM. Other conditions of the assay were the same as for determination of AlAT activity. The results are mean values, ±S.D., from 4 experiments with bicarbonate buffer, and 3 experiments with pyrophosphate buffer.

Source of enzyme	Buffer	Alanine	2-Oxo-glutarate	Glutamate	Pyruvate
		K_m (mM)			
Rat brain cytoplasm	pyrophosphate (50 mM)	32.8±4.0	1.30±0.29	16.3±1.5	1.10±0.12
	bicarbonate (60 mM)	9.1±1.5[a]	0.71±0.05[b]	8.0±1.0[a]	0.33±0.05[a]
mitochondria	bicarbonate (60 mM)	4.3±1.0	0.63±0.20		
	bicarbonate (40 mM)	6.1±1.5	0.54±0.10	12.7±1.5	0.38±0.05
Rat liver mitochondria	pyrophosphate (50 mM)	13.3±1.0	1.70±0.20		
	bicarbonate (40 mM)	6.1±0.7[a]	0.50±0.30[c]		

[a] $p<0.001$.
[b] $0.02<p<0.05$.
[c] $0.001<p<0.005$.

For the enzyme from brain mitochondria the K_m value for alanine was half that obtained with the cytoplasmic enzyme at the same concentration of bicarbonate buffer (60 mM). The lowering of concentration of this buffer from 60 to 40 mM resulted in a slight increase of K_m for alanine from 4.3 to 6.1 mM, whereas it had practically no effect on the K_m value for 2-oxoglutarate.

DISCUSSION

The results show that bicarbonate exerts different effects on aminotransferases. Under the experimental conditions used, AspAT was markedly inhibited, whereas AlAT was stimulated by bicarbonate. The rate of the reaction catalysed by AlAT was accelerated both with the increase in CO_2 pressure and in bicarbonate concentration.

The activity of cytoplasmic rat brain alanine aminotransferase was more affected by bicarbonate than that of brain and liver mitochondrial enzyme.

The K_m values of the brain and liver mitochondrial AlAT for alanine and 2-oxoglutarate, at a given bicarbonate concentration and pH value, were similar. This, together with the similar response to such inhibitors as aminooxyacetic acid (Hopper & Segal, 1964) and keto derivatives of branched-chain amino acids (Łysiak *et al.*, 1974) support identity of AlAT enzymes from both tissues studied. The K_m values determined in bicarbonate buffer were much lower than those obtained in pyrophosphate buffer (Hopper & Segal, 1962; Swick *et al.*, 1965; and the present work).

It may be supposed that stimulation of AlAT by bicarbonate buffer might be due to the increased affinity of the enzyme for its substrates, similarly as it was suggested for stimulation of AlAT by formate (Saier & Jenkins, 1967b). Bicarbonate anion might change conformation of the enzymatic protein due to binding with the subsite within the active region of the enzyme. According to Morino & Okamoto (1972) this subsite normally binds the second carboxylate groups of 4 - 5 carbon amino acids, such as aspartate. This assumption is in agreement with the inhibition of aspartate aminotransferase by bicarbonate, observed in this work.

It seems that regulation of AlAT activity by bicarbonate might be of significance *in vivo*. The intracellular pH value in brain tissue is 7.04 at a CO_2 pressure of 45 mm Hg (Siesjö *et al.*, 1972). Under these conditions, AlAT shows the highest sensitivity to variation in the HCO_3^-/pCO_2 ratio. In respiratory acidosis or alkalosis the content of high-energy compounds is unaltered whereas changes are observed in the level of the intermediates of glycolysis and the Krebs cycle, and in the pool of the amino acids related to this cycle (Folbergrova *et al.*, 1972a,b; MacMillan & Siesjö, 1972; Granholm *et al.*, 1969). As suggested by Folbergrova *et al.* (1972b), these changes are associated with the response of such enzymes as phosphofructokinase, isocitrate dehydrogenase and lactate dehydrogenase to variation in the intracellular pH. Presumably, alanine aminotransferase is also susceptible to changes in pH, which in consequence affect alanine synthesis.

The high rate of alanine synthesis in brain is evidenced by a much more rapid labelling of alanine, as compared with other amino acids, following intravenous infusion of [^{14}C]glucose (Stone *et al.*, 1972). Alanine is known to be one of the amino acids which hardly pass the blood/brain barrier (Oldendorf, 1971). The ratio of alanine concentration in brain tissue and blood is close to unity (Battistin *et al.*, 1971), while large differences occur in the content of this amino acid in arterial and venous blood (Felig & Wahren, 1971). This observation may suggest that, despite rapid synthesis, alanine does not accumulate in brain; possibly it may play the role of an amino group carrier in the so-called alanine cycle (Mallette *et al.*, 1969). In brain, the cytoplasmic fraction contains about 90% of the AlAT activity (Benuck *et al.*, 1972) and is predominantly responsible for the synthesis of alanine in this tissue. As it may be judged from concentration of the substrates (Duffy *et al.*, 1972), the reaction catalysed by AlAT *in vivo* is not in a state of equilibrium in brain (Duffy *et al.*, 1972), in contrast to liver (Williamson *et al.*, 1967). Since the equilibrium constant is close to unity, the direction of the transamination reaction is dependent to a large extent on the concentration of substrates. This is probably the reason why in hypoxia the increased alanine content results from the increase in pyruvate

concentration (Duffy *et al.*, 1972). It seems that the second factor controlling the rate of alanine synthesis *in vivo* is the concentration of bicarbonate anion which affects the affinity of AlAT for the substrates. The K_m value for glutamate determined in bicarbonate buffer corresponds to the concentration of this compound in brain tissue; for pyruvate this value is three times as high, and for alanine and 2-oxoglutarate ten times as high as the concentration of these compounds in brain (Folbergrova *et al.*, 1972b; Miller *et al.*, 1973). This implies that under conditions favouring acidosis and raised concentration of intracellular HCO_3^-, alanine synthesis would be stimulated.

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AKTYWNOŚĆ CYTOPLAZMATYCZNEJ I MITOCHONDRIALNEJ AMINOTRANSFERAZY ALANINOWEJ W BUFORZE DWUWĘGLANOWYM

Streszczenie

1. Bufor dwuwęglanowy stymuluje aktywność aminotransferazy alaninowej (EC 2.6.1.2) z cytoplazmy mózgu szczura oraz zwiększa powinowactwo substratów do enzymu cytoplazmatycznego i mitochondrialnego, wyrażające się obniżeniem wartości K_m.

2. Aktywność krystalicznej aminotransferazy asparaginowej (EC 2.6.1.1) jest w tych samych warunkach hamowana przez bufor dwuwęglanowy.

3. Przedyskutowano rolę jonu dwuwęglanowego w regulacji aktywności aminotransferazy alaninowej i syntezy alaniny.

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ISOLATION, PURIFICATION AND CHEMICAL COMPOSITION OF INSOLUBLE COLLAGEN FROM GUERIN EPITHELIOMA

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1. The insoluble collagen from Guerin epithelioma was isolated and its chemical composition was determined. The unusually high histidine content is accompanied in tumour collagen by a relatively small amount of lysine and arginine.

2. The isolated protein was strongly bound to glycoprotein, which could not be removed by EDTA treatment unless this procedure was preceded by digestion of the complex with trypsin.

Native insoluble collagen is usually bound to various types of glycoproteins, glycosaminoglycans and proteoglycans of connective tissue (Steven, 1967; Schofield *et al.*, 1971; Anderson & Jackson, 1972; Miller, 1972; Kobayashi & Pedrini, 1973; Öbrink, 1973). In epithelial tumours, neoplastic cells are accompanied by connective tissue and collagen fibres, therefore isolation and characterization of insoluble collagen from epitheliomas seems of interest.

It has been previously found that collagen of Guerin epithelioma is strongly resistant to EDTA (Bańkowski *et al.*, 1974), in contrast to collagen derived from other tissues (Steven, 1967; Schofield *et al.*, 1971). Digestion of native tumour collagen with bacterial collagenase caused its degradation to low-molecular products (Bańkowski *et al.*, unpublished data). It has also been suggested (Bańkowski *et al.*, 1974) that resistance to EDTA is due to the large amount of other proteins strongly bound to collagen in tumour tissue. Since Worrall & Steven (1966) and Steven & Thomas (1973) demonstrated that native collagen is not hydrolysed by trypsin at low temperature, we have used this enzyme to remove the contaminating collagen-bound proteins. The applied procedure made it possible to remove most of the non-collagenous material and rendered the insoluble collagen susceptible to the EDTA treatment.

MATERIAL AND METHODS

Tumour growth. The Guerin epitheliomas were grown on Wistar rats as described by Bańkowski *et al.* (1974); for collagen preparation, 40-day-old tumours were used.

Purification of insoluble collagen. About 300 mg of collagen partially purified as described by Bańkowski *et al.* (1974), was digested with 30 ml of 0.2% trypsin (9000 units/mg, Koch-Light Lab. Ltd, Colnbrook, Bucks., England) dissolved in 1% $NaHCO_3$, at 18°C for 24 h with occasional stirring. The suspension was centrifuged at 20 000 *g* for 20 min. The supernatant was discarded and digestion of the sediment with trypsin was repeated. The insoluble protein was separated by centrigufation and washed twice with water and then treated with EDTA, pH 7.4, to a final concentration of 4%, at room temperature for 24 h with occasional stirring. The insoluble protein was separated by centrifugation and extracted repeatedly with 0.2 M-acetic acid at 4°C for 3 days with permanent shaking, the extract being removed every day. By this procedure, total solubilization of the collagen was achieved. The acetic acid extracts were combined, clarified by ultracentrifugation (100 000 *g* for 1 h) and the dissolved collagen was precipitated by addition of NaCl to a final concentration of 5% (w/v). The precipitate was redissolved in 0.2 M-acetic acid and reprecipitated by raising pH to 6 with 1 M-NaOH. The solubilization and reprecipitation procedure was repeated and the purified collagen was dissolved in 0.2 M--acetic acid. The collagen solution was dialysed exhaustively against 0.2 M-acetic acid to remove the contaminating salts, then it was freeze-dried.

Molecular-sieve chromatography. To the solution of tumour collagen in 0.2 M-acetic acid, solid sodium dodecyl sulphate (SDS) was added to 1% concentration, and the mixture was heated in a boiling-water bath for 2 min. A 7 ml aliquot was applied to a Sephadex G-200 column (42 × 3.2 cm) equilibrated with 0.2 M-acetic acid containing 0.2% SDS, and collagen was eluted with the same mixture at a rate of 20 ml/h. Fractions of 10 ml were collected, in which hydroxyproline content was estimated.

Gel chromatography of the trypsin digestion products of collagen-bound substance was performed on the Sephadex G-200 column (42 × 3.2 cm) which in this case was equilibrated with 1% $NaHCO_3$. A 7 ml sample of the trypsin digest was applied to the column; fractions of 5 ml were collected at a rate of 20 ml/h, and absorbance at 280 nm was monitored.

Nitrogen content was determined by the method of Kjeldahl.

Amino acid analysis. Protein was hydrolysed in 6 M-HCl at 120°C for 18 h and the amino acids were analysed using a Beckman autoanalyser (Unichrom). Hydroxyproline was estimated by the method of Prockop & Udenfriend (1960).

Determination of hexose, hexosamine and uronic acids. Hexoses were identified and quantitatively estimated by gas-liquid chromatography. The instrument used was Beckman (model GC 2) chromatograph equipped with a flame ionization detector and a Honeywell-Brown 0-1 mV recorder. The stainless steel column (0.4 × × 186 cm) was packed with 3% SE-30 on chromosorb W AW DMCS, 80 - 100

mesh. The analyses were made at 150°C (isothermally) with nitrogen as a carrier gas at a pressure of 2.1 atm. Samples of protein were methanolysed, using mannitol as an internal standard (Bhatti *et al.*, 1970) and the dry material was dissolved in 1 ml of dimethylformamide. The obtained solutions were transferred to small tubes and 0.3 ml of hexamethyldisilazane and 0.2 ml of trimethylchlorsilane were added. The tubes were sealed with polyethylene caps and after 24 h samples of the upper phase, 1 - 5 µl, were injected into gas chromatograph (Ellis, 1969). The results were calculated as described by Bhatti *et al.* (1970).

For the hexosamine assay, hydrolysis of the investigated material was performed in 4 M-HCl at 100°C for 12 h. Hexosamines (glucosamine and galactosamine) were separated from hydrolysate by the method of Głowacka *et al.* (1967) and estimated as described by Ludowieg & Benmaman (1967).

Uronic acids were determined according to Bitter & Muir (1962) with D-glucuronic acid as a standard.

RESULTS

The insoluble collagen of Guerin epithelioma could not be purified by EDTA (Table 1) as the preparation obtained contained only 2.42% of hydroxyproline. Typical collagen contains 12 - 14% (w/w) of this imino acid (Banga, 1966; Reich, 1966). This points to heavy contamination of the collagen preparation from epithelioma with non-collagenous compounds.

Most of the collagen-bound substances could be removed by trypsin digestion (Table 1), and the insoluble residue obtained contained 9.77% of hydroxyproline. It swelled and gelatinized in acidic solutions very well. The trypsin-treated material became sensitive to EDTA treatment, and by the latter procedure full solubilization

Table 1

Hydroxyproline content and the hydroxyproline : nitrogen ratio at the successive stages of purification of insoluble collagen from Guerin epithelioma

For comparison, the data for highly purified lathyric rat skin collagen are included.

Stage of preparation	Hydroxyproline (% of dry wt.)	Hydroxyproline/ nitrogen ratio (mg/mg)
Homogenate	0.33	0.024
EDTA treatment + extraction with acetic acid	2.42	0.180
Trypsin digestion	9.77	0.674
IInd EDTA treatment + extraction with acetic acid (final preparation)	12.44	0.718
Lathyric rat skin collagen*	13.50	0.781

* Kindly supplied by Dr Hector Aquilar, Department of Biochemistry, Vanderbilt University, Nashville, U.S.A.

of insoluble collagen was achieved. The final product contained 12.44% of hydroxyproline and the hydroxyproline : nitrogen ratio was similar to that of collagen from other sources (Gustavson, 1956).

Since the tumour collagen was eluted close to the void volume of Sephadex G-200 column, it may be concluded that this protein was not degraded during trypsin digestion. The collagen-bound protein, hydrolysed by trypsin to low-molecular material, was eluted on exhaustive washing (Fig. 1).

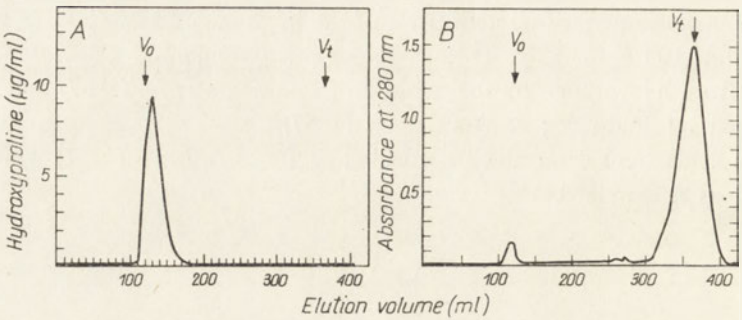

Fig. 1. Sephadex G-200 column chromatography of collagen of Guerin epithelioma (*A*), and of the collagen-bound substances digested by trypsin (*B*).

Table 2

Amino acid composition of insoluble collagen of Guerin epithelioma

For comparison, the data of Kao *et al.* (1968) for collagen of rat uterus are included.

Amino acid	Epithelioma	Rat uterus
	residues/1000 residues	
Aspartic acid	44.8	49
Threonine	9.9	17
Serine	17.5	39
Glutamic acid	49.6	77
Proline	122.2	113
Glycine	374.0	340
Alanine	38.2	101
Cysteine	15.2	0
Valine	0.0	22
Methionine	0.0	5
Isoleucine	12.9	11
Leucine	22.9	24
Tyrosine	0.0	4
Phenylalanine	0.0	12
Lysine	13.4	18
Histidine	159.5	5
Arginine	29.7	52
Hydroxyproline	80.0	102
Hydroxylysine	7.9	16
Tryptophan	not determined	

The insoluble collagen of Guerin epithelioma contained proline, hydroxyproline and glycine in the amounts characteristic for this protein, and contained no aromatic amino acids (Table 2). However, it was found to contain a slight amount of cysteine and an unusually high histidine content; other basic amino acids were present in rather small amounts. As Guerin epithelioma origins from rat uterus, the data of Kao *et al.* (1968) on the amino acid composition of insoluble collagen from this tissue are included in Table 2.

Table 3

Carbohydrate composition of insoluble collagen of Guerin epithelioma (A) and of the collagen-bound substances digested by trypsin (B)

The results are expressed as percentages of dry weight.

Carbohydrate	A	B
Glucose	0.75	1.61
Galactose	0.30	0.90
Glucosamine	0.00	0.57
Galactosamine	0.00	trace
Uronic acids	0.00	0.00

The carbohydrate composition of the purified tumour collagen is presented in Table 3. Glucose and galactose were found, and hexosamines and uronic acids were not detected. Some carbohydrate compounds (glucose, galactose, mannose and glucosamine) were also found in the collagen-bound substances released by trypsin digestion (Table 3).

DISCUSSION

The occurrence of hydroxyproline is limited to a few proteins, such as collagen and elastin. The amount of hydroxyproline in collagen of mammalian connective tissue is very stable (about 13%, w/w). For this reason the hydroxyproline content and/or the hydroxyproline : nitrogen ratio is a good index of collagen purity (Gustavson, 1956; Banga, 1966; Reich 1966). It is well known that all types of substances accompanying collagen, like glycoproteins, glycosaminoglycans and proteoglycans, contain hexosamines (Schofield *et al.*, 1971; Hanada & Anan, 1973; Kempson *et al.*, 1973; Herbage *et al.*, 1974), therefore the lack of glucosamine and galactosamine in a collagen preparation is recommended as the best criterion of purity of this protein (Schofield *et al.*, 1971; Grant & Jackson, 1968). According to these criteria, the preparation of collagen from Guerin epithelioma obtained in this work, can be considered to be pure.

The amino acid composition of our purified preparation was similar to that of other types of collagen, except that the content of histidine was unusually high. Besides, the preparation contained cysteine, the presence of which has been reported only for collagen of glomerular basement membrane in kidney (22.8/1000 residues;

Westberg & Michael, 1970). The glucose : galactose ratio in tumour collagen was higher than in typical collagen of connective tissue (Schofield *et al.*, 1971).

Since digestion with trypsin resulted in the release of low-molecular products and about 4% of carbohydrates, one may assume that the insoluble collagen of Guerin epithelioma is strongly bound to glycoprotein: 1 mg of tumour collagen binds about 4 mg of glycoprotein (as calculated from Table 1). This glycoprotein may be responsible for the resistance of native collagen to EDTA and for its lack of swelling and gelatinization. Such stable complexes of collagen with a large amount of glycoprotein were not found in any normal tissue.

As this complex did not dissociate in concentrated urea or guanidine-HCl solutions (Bańkowski *et al.*, unpublished results), the bonding between collagen and glycoprotein is presumably of covalent nature.

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IZOLOWANIE, OCZYSZCZANIE I SKŁAD CHEMICZNY NIEROZPUSZCZALNEGO KOLAGENU Z NABŁONIAKA GUERIN

Streszczenie

1. Oczyszczono nierozpuszczalny kolagen z nabłoniaka Guerin i oznaczono jego skład chemiczny. Stwierdzono wysoką zawartość histydyny przy stosunkowo niskiej zawartości lizyny i argininy.

2. Badany kolagen jest silnie związany z glikoproteidem. Kompleks ten jest oporny na działanie EDTA i poddaje się jego działaniu dopiero po trawieniu trypsyną.

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MITOCHONDRIA FROM THE LAMPREY (*LAMPETRA FLUVIATILIS*). OXIDATIVE PHOSPHORYLATION AND RELATED PROCESSES

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1. High efficiency of oxidative phosphorylation and a good respiratory control in liver, heart and somatic muscle mitochondria of the lamprey (*Lampetra fluviatilis*) were observed when the particles were isolated in a complex sucrose medium containing EDTA, heparin and nicotinamide. The coupling properties of these mitochondria were further improved by including serum albumin in the incubation medium.

2. The content of total adenine nucleotides in lamprey mitochondria was between 4 and 6 nmoles/mg protein. The translocation of these nucleotides across mitochondrial membrane was stimulated by serum albumin.

3. Lamprey mitochondrial phospholipids contain a large proportion (64 - 72%) of polyunsaturated fatty acids.

4. Electron micrographs of mitochondria from lamprey liver, heart and somatic muscle are presented.

The metabolism and properties of fish mitochondria have been investigated in several species and organs (for reviews see Hochachka, 1969; Hochachka & Somero, 1973). Contrary to this, little information is available on the energy metabolism of lower vertebrates, e.g. *Cyclostomata*. Among them, lamprey (*Lampetra fluviatilis*) is of particular interest because of a long period of physiological starvation of mature animals. Sexually mature lampreys migrate from the sea to rivers in the autumn and take no food till they die after spawning next spring (Hardisty & Potter, 1971).

In a preliminary report (Savina & Kudryavtseva, 1973) it was shown that the rate of oxidation of several respiratory substrates by mitochondria from lamprey heart and somatic muscles was comparable to that by mitochondria from rat and pigeon heart and skeletal muscles. However, the efficiency of oxidative phosphory-

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lation by lamprey mitochondria was very low. The aim of the present work was to describe in more detail some bioenergetic features of lamprey mitochondria during winter starvation. By improving isolation and incubation conditions more tightly coupled mitochondria from lamprey tissues were obtained.

MATERIALS AND METHODS

Lampreys (*Lampetra fluviatilis*) were caught between November and March in the lower Vistula River and in estuaries of the Baltic Sea and were kept in the laboratory in running tap water of about 5°C for several weeks. The animals were killed by decapitation and liver, heart and somatic muscles were excised. This step and all further manipulations were carried out at 0 - 4°C. The tissues were cut into small pieces and homogenized in a glass-teflon homogenizer with a tenfold volume of the isolation medium. Preliminary experiments showed that mitochondria with the highest respiratory control ratio were obtained in the following isolation medium which was therefore used in all subsequent experiments: 250 mM-sucrose, 2 mM-Tris-HCl (pH 7.4), 1 mM-EDTA, 30 mM-nicotinamide, and heparin, 50 units/ml. The homogenates were centrifuged 10 min at 700 - 800 *g* and the resulting supernatants were centrifuged 15 min at 16 000 *g* to sediment mitochondria. The mitochondria were subsequently washed twice by resuspending in the same medium and sedimenting by centrifugation at 16 000 *g* for 10 - 15 min.

Oxygen uptake was measured polarographically using a Clark type electrode. The medium was: 20 mM-KCl, 50 mM-sucrose, 6 mM-$MgCl_2$, 4 mM-EDTA, 20 mM-Tris-HCl and 20 mM-Na-K-phosphate (pH 7.4). Total volume was 3.0 ml and the amount of added mitochondria corresponded to 3 - 4 mg protein. Temperature was 25°C.

The total amount of mitochondrial adenine nucleotides was determined by the enzymatic procedure (Williamson & Corkey, 1969). The nucleotides were extracted from mitochondria by 1.5 M-perchloric acid, the extract was neutralized with $KHCO_3$, potassium perchlorate was centrifuged off, and the adenine nucleotides were phosphorylated to ATP using phosphoenolpyruvate, pyruvate kinase (EC 2.7.1.40) and adenylate kinase (EC 2.7.4.3).

Translocation of adenine nucleotides was measured by the inhibitor-stop method as described by Wojtczak & Załuska (1967) and modified by Duszyński & Wojtczak (1974). The medium contained 120 mM-KCl, 10 mM-Tris-HCl (pH 7.4) and 3 mM-$MgCl_2$. The reaction was started by addition of [^{14}C]ADP to the final concentration of 70 μM and stopped by 5 μM-carboxyatractyloside. The temperature was 0°C.

To determine fatty acid composition, mitochondrial lipids were extracted by the method of Folch *et al.* (1957) and free fatty acids were separated from fatty acid esters (mostly phospholipids) by the procedure of Borgström (1952). The esters were hydrolysed and methanolysed by heating them at 80 - 90°C for 1 hour in methanol containing 0.5 M-KOH. Complete methylation of fatty acids was obtained by refluxing for 2 hours in anhydrous HCl in methanol as described by James (1960). Methyl esters were then chromatographed in a Pye 104 Gas Chromatograph

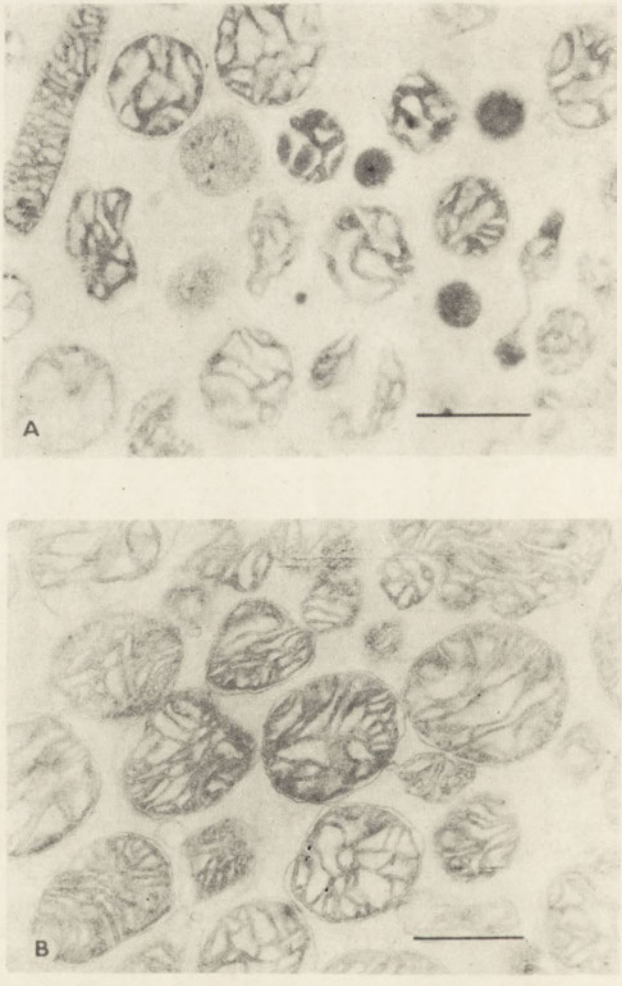
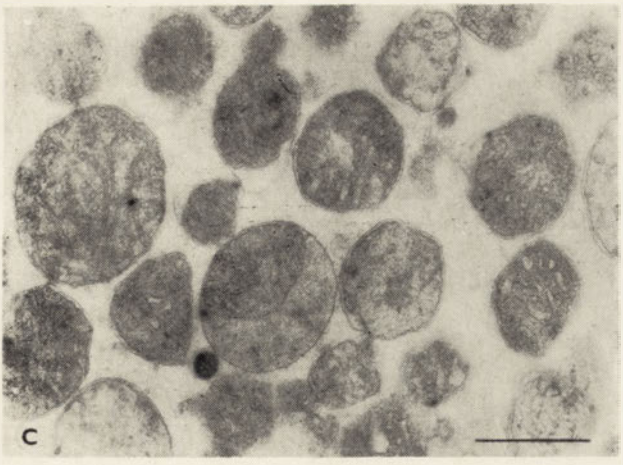
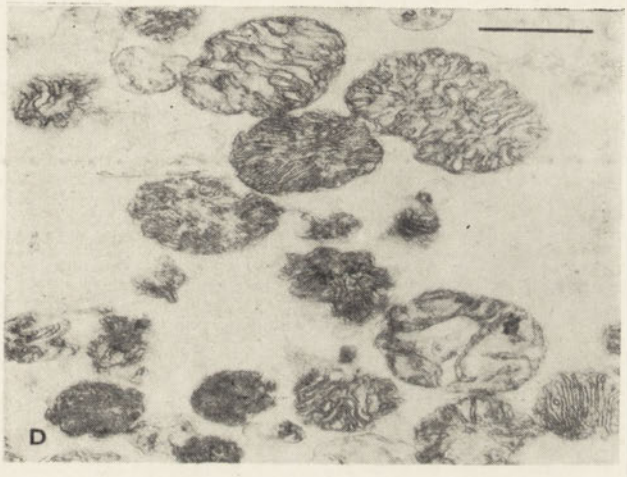


Fig. 1. Electron micrographs of lamprey liver, heart and somatic muscle mitochondria. (A) Liver and (B) heart mitochondria fixed in form of pellets sedimented from the sucrose isolation medium (see Materials and Methods); (C) liver, (D) heart and (E) somatic muscle mitochondria fixed as suspension in 120 mM-KCl - 10 mM-Tris-HCl (pH 7.4). The bars represent 1 μm.





operated isothermally at 200°C, with argon as the carrier gas. A 5 ft. column of 10% polyethylene glycol adipate on 100 - 120 mesh Diatomite C was used for the separation.

For electron microscopy, mitochondria were fixed either in suspension or in pellet using 1% (final concentration) osmium tetroxide in 0.1 M-cacodylate buffer (pH 7.4). They were dehydrated in ethanol and propylene oxide, embedded in Epon and sliced on Tesla model BS 490A ultramicrotome. Slices were stained with uranyl acetate and lead citrate and examined in JAM 7A electron microscope (Japan Electron Optics Laboratories Co., Tokyo) at 80 kV.

Protein was determined by the biuret method (Gornall *et al.*, 1949).

RESULTS

Table 1 shows the amount of mitochondria which could be isolated by the procedure used in the present investigation from various tissues of the lamprey. It can also be seen that membrane proteins contribute to about 50% of total protein in liver mitochondria whereas they amount to about 75% in mitochondria from heart and somatic muscles.

Table 1

Yield of mitochondria isolated from various tissues of the lamprey and the content of membraneous and soluble mitochondrial proteins

The values are from 5 to 9 experiments. Both mean values and the range (in parentheses) are shown. To determine proportions between membraneous and soluble proteins, mitochondria were sonicated and centrifuged for 1 hour at 100 000 *g*.

Tissue	Yield of mitochondria (mg mitochondrial protein/g fresh tissue wt.)	Mitochondrial proteins	
		membraneous (%)	soluble (%)
Liver	6 (4 - 7)	50	50
Heart	15 (11 - 18)	70 - 75	25 - 30
Somatic muscles	2 (1.6 - 3.4)	70	30

Electron microscopy. When the pellets of freshly isolated mitochondria in sucrose solution were fixed, a strongly condensed configuration was observed (Fig. 1 A and B). Intact outer membranes were clearly visible in most of the profiles. When mitochondria were suspended in 120 mM-KCl - 10 mM-Tris-HCl (pH 7.4) the matrix space became expanded. In liver mitochondria (Fig. 1 C), occasionally "orthodox" configuration could be observed, but in most of the particles the swelling proceeded further resulting in swollen profiles. In heart and somatic muscle mitochondria (Fig. 1 D and E), profiles of more or less "orthodox" configuration appeared after suspending the pellet in 120 mM-KCl. "Orthodox" mitochondria of liver, heart

and somatic muscles of the lamprey resemble mammalian liver, heart and skeletal muscle mitochondria, respectively (cf. Lehninger, 1964).

Respiration and phosphorylation. Preliminary experiments showed that lamprey liver mitochondria isolated in 250 mM-buffered sucrose had rather low respiratory activity with all tested respiratory substrates and no respiratory control upon addition of ADP or uncouplers of oxidative phosphorylation. It was supposed that the lack of respiratory control might result from the presence of non-esterified fatty acids, similarly as is the case with mitochondria isolated from some insects (Wojtczak & Wojtczak, 1960; Wojtczak *et al.*, 1968; for reviews on the uncoupling action of fatty acids see Wojtczak *et al.*, 1969; Wojtczak, in press). To verify this assumption, the following substances were included in the isolation and/or the incubation media: (a) EDTA known to inhibit lipolysis by chelating Ca^{2+} (Wills, 1960; Waite & van Deenen, 1967); (b) nupercaine, an inhibitor of phospholipases (Scherphof *et al.*, 1972), which preserves functional (Scarpa & Lindsey, 1972) and structural (Aleksandrowicz *et al.*, 1973) integrity of mitochondria; (c) heparin which inhibits lipoprotein lipases acting on endogenous lipoproteins (Korn, 1962); and (d) serum albumin known to bind fatty acids (Goodman, 1958). Besides, nicotinamide was also included in the isolation medium to inhibit NADase (NAD glycohydrolase, EC 3.2.2.5) as recommended by Brown & Tappel (1959) for carp liver mitochondria and by Devlin (1967) for preparation of mitochondria from neoplastic tissues.

With 1 mM-EDTA and 30 mM-nicotinamide in the isolation medium the respiratory control of lamprey liver mitochondria with succinate, glutamate and 2-oxoglutarate was about 2, provided serum albumin (10 mg/ml) was present in the incubation medium. Nupercaine at the concentration of 0.4 mM improved the coupling of mitochondria; however, a substantial improvement was obtained on addition of heparin (50 units/ml) to the isolation medium. The use of heparin has first been recommended for the separation of mammalian skeletal muscle mitochondria by Dow (1967) and Max *et al.* (1972). In lamprey liver mitochondria isolated in the presence of heparin, respiratory control could be observed even without serum albumin. Typical oxygraphic traces are shown in Fig. 2.

Table 2 summarizes the results obtained with mitochondria from lamprey liver and muscles with various respiratory substrates. Although the respiratory control was relatively low, as compared e.g. to mammalian liver and muscle mitochondria, ADP/O ratios were high, approaching theoretical values. It is also evident from Table 2 that the oxidation of glutamate and 2-oxoglutarate was accompanied by substrate-level phosphorylation, as indicated by ADP/O ratios substantially exceeding 3.

Content and transport of adenine nucleotides. Preliminary enzymatic determinations of the content of the sum of adenine nucleotides (ATP+ADP+AMP) in lamprey mitochondria revealed the following values (in nmoles/mg protein): for liver mitochondria, 4.4 and 5.9 (two determinations); for heart mitochondria, 3.9; and for somatic muscle mitochondria, 5.2 (single determinations).

Experiments with the exchange of adenine nucleotides between the inner mitochondrial compartment and the external medium showed that the isotopic equilibrium

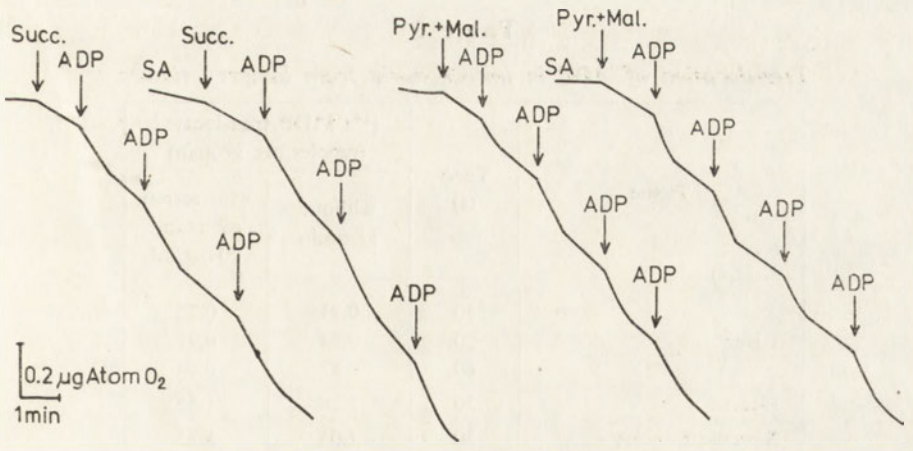


Fig. 2. Respiratory activity of lamprey liver mitochondria isolated in 250 mM-sucrose containing EDTA, heparin and nicotinamide. Each sample contained 4.2 mg mitochondrial protein. Additions to the incubation medium: succinate (Succ.), 5 mM; pyruvate *plus* malate (Pyr.+Mal.), 5 mM each; ADP, 167 µM (0.5 µmole); serum albumin (SA), 10 mg/ml.

Table 2

Respiration and phosphorylation in mitochondria from lamprey liver and somatic muscles

Respiratory substrates were used at final concentrations of 5 mM. For state 3 respiration, 167 µM-ADP was added. State 4 respiration was measured when added ADP was completely phosphorylated (for the definition of respiratory states 3 and 4 see Chance & Williams, 1956).

Respiratory substrate	Bovine serum albumin* (10 mg/ml)	Liver mitochondria				Somatic muscle mitochondria			
		Oxygen uptake (ngAtom/mg protein/min)		Respiratory control	ADP/O	Oxygen uptake (ngAtom/mg protein/min)		Respiratory control	ADP/O
		State 3	State 4			State 3	State 4		
Endogenous	+		16				15		
Succinate	—	63	27	2.3	1.8				
Succinate	+	71	31	2.3	1.7	126	52	2.4	1.9
Malate	+					178	38	4.7	2.4
Pyruvate+malate	—	69	29	2.4	2.3	131	44	3.0	2.8
Pyruvate+malate	+	80	24	3.3	2.6	155	37	4.2	2.8
Palmitoylcarnitine	+					59	30	2.0	2.3
2-Oxoglutarate	+					113	33	3.4	3.1
Glutamate+malate	+					92	37	2.5	3.4

* Fraction V from Sigma Chemical Co (St. Louis, Mo., U.S.A.).

was attained at 0°C after 60 s. Therefore, shorter incubation time was applied to study the rate of exchange. For liver mitochondria it was found (Table 3) that the exchange measured after 10 s and 20 s was considerably increased by serum albumin, but the final level reached after 60 s, corresponding to the exchangeable pool of ade-

Table 3

Translocation of ADP in mitochondria from lamprey tissues

Tissue	Time (s)	[^{14}C]ADP translocated (nmoles/mg protein)	
		without albumin	with serum albumin, 20 mg/ml
Liver	10	0.31	0.75
	20	0.34	0.91
	60	0.87	0.88
Heart	30	1.28	1.35
Somatic muscles	30	1.05	1.22

nine nucleotides (ATP+ADP), was the same with and without serum albumin. The stimulatory effect of serum albumin in mitochondria from heart and somatic muscles was less pronounced. Experiments not shown in Table 3 revealed that preincubation of mitochondria with oxoglutarate at room temp. for 10 min increased the exchangeable pool of adenine nucleotides by about 50% in liver mitochondria and by about 10% in heart mitochondria. The final values for this pool varied then between 1.2 and 1.6 nmoles/mg protein.

Table 4

Fatty acid composition of lipids in mitochondria from liver, heart and somatic muscles of the lamprey

The data are expressed as percentage of total fatty acids. EFA, esterified fatty acids (mostly phospholipids); NEFA, non-esterified fatty acids; traces, less than 1%.

Fatty acid*	Mitochondria from					
	liver		heart		somatic muscles	
	EFA	NEFA	EFA	NEFA	EFA	NEFA
14:0	2	2	traces	2	1	2
16:0	9	17	8	10	11	13
16:1	2	2	2	3	3	3
18:0	4	10	5	11	4	8
18:1	21	20	15	21	15	17
18:2	6	11	9	10	11	14
18:3	1	2	2	3	3	4
20:2	1	3	1	2	2	2
20:3	5	4	3	4	3	3
20:4	traces	0	0	0	1	0
20:5	15	12	10	10	13	11
22:5	3	0	2	0	2	2
22:6	33	17	45	24	31	21

* The first number indicates the number of carbon atoms, the second one the number of double bonds.

Fatty acid composition of mitochondrial phospholipids. Because of the well known highly unsaturated character of storage lipids in lamprey it was of interest to see whether this had any reflection in fatty acid composition of phospholipid constituents of mitochondrial membranes. To examine this point, mitochondrial lipids were extracted, fractionated into esterified and non-esterified fatty acids, and examined by gas chromatography. It can be safely assumed that the fraction of esterified fatty acids mostly represents phospholipids since these compounds mainly contribute to mitochondrial lipids.

It can be seen (Table 4) that fatty acid composition of the esterified fraction is similar in mitochondria from all three tissues under investigation. The main constituents are palmitic ($C_{16:0}$), oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) acids, as well as $C_{20:5}$ and $C_{22:6}$ acids. The percentage of the last acid appeared particularly high, amounting in heart mitochondria to 45% of total esterified fatty acids.

The absolute amount of non-esterified fatty acids was low and was not determined quantitatively. Nevertheless, the composition of this fraction was similar to that of esterified fatty acids (Table 4), with the exception that the sum of C_{18} acids was somewhat higher and the proportion of the $C_{22:6}$ acid distinctly lower in the non-esterified than in the esterified fraction.

DISCUSSION

The present investigation shows that mitochondria isolated from lamprey liver, heart and somatic muscles are tightly coupled, provided lipolytic processes are sufficiently inhibited during the isolation procedure. This suggests that these mitochondria are normally coupled *in situ* and their poor coupling observed after isolation in plain sucrose, especially evident in the case of liver mitochondria, was due to liberation of fatty acids during the isolation procedure.

It may be of interest to mention here that, according to our recent preliminary observations (Savina, unpublished), liver mitochondria from lampreys caught during early autumn (September and October) showed good respiratory control even when isolated without heparin or nupercaine, provided serum albumin was present in the incubation medium. A seasonal dependence of the efficiency of oxidative phosphorylation has also been observed previously with mitochondria from lamprey heart and somatic muscles (Savina & Kudryavtseva, 1973). Thus, a deterioration of coupling properties of isolated lamprey mitochondria, which becomes apparent during the last winter of the animal's life, may be related to profound atrophic changes occurring in its organs at that time (Hardisty & Potter, 1971). We have also observed (Savina, unpublished) a drastic change in the proportion of adenine nucleotides in the whole lamprey liver between October (high ATP, low AMP) and December (low ATP, high AMP).

Effects of fatty acids on energy coupling processes in mitochondria have often been observed. Fatty acids are known to stimulate mitochondrial ATPase (EC 3.6.1.3) (Pressman & Lardy, 1956), and to lower P/O ratio (Hülsmann *et al.*, 1960; Wojtczak & Wojtczak, 1960; Borst *et al.*, 1962). They also increase the permeability

of mitochondrial membranes to monovalent cations (Wojtczak, 1974) and passive (carrier-independent) permeability to phosphate (Wojtczak, in press). Thus, fatty acids produce, even at low concentrations, strong alterations in properties of mitochondrial membranes (for review see Wojtczak, in press). Among various fatty acids the most effective in this respect are saturated acids of medium chain-length and unsaturated long-chain acids (Zborowski & Wojtczak, 1963; Wojtczak, 1974). Therefore it is important to note that lamprey mitochondria contain a high proportion of polyunsaturated long-chain fatty acids.

Mitochondria from lamprey tissues contain about half of the amount of adenine nucleotides as compared to mammalian liver (Ernster *et al.*, 1967) and heart (LaNoue *et al.*, 1972) mitochondria. At the present state of investigation it cannot be decided whether this relatively low content of these nucleotides is intrinsic to lamprey mitochondria *in situ* or is the result of some leakage during isolation of mitochondria. When mitochondrial adenine nucleotides were determined by equilibration with external radioactive ADP still much lower values were obtained. Even after preincubation with 2-oxoglutarate, that is under conditions expected to convert intramitochondrial AMP into ADP and ATP (Rossi *et al.*, 1971) the amount of exchangeable nucleotides did not exceed 1.6 nmoles/mg protein. The explanation of this discrepancy between direct enzymatic determination and that calculated from isotopic equilibration is not clear. It might be speculated that either the conversion of AMP (not exchangeable) to ADP and ATP (exchangeable with external ADP) during preincubation with 2-oxoglutarate is not complete, or that a leakage of adenine nucleotides occurs during measurement of the translocation.

The stimulatory effect of serum albumin on adenine nucleotide translocation in lamprey mitochondria is similar to that observed by Christiansen *et al.* (1973) in brown adipose tissue mitochondria and points to the inhibition of this translocation by fatty acids (Wojtczak & Załuska, 1967; Wojtczak *et al.*, 1969) and/or long-chain acyl-CoA (Pande & Blanchaer, 1971; Lerner *et al.*, 1972).

An interesting feature of lamprey muscle mitochondria is their high respiratory activity with malate as the only substrate (Table 2). As the equilibrium for the reaction

$$\text{malate} + \text{NAD} \rightleftharpoons \text{oxaloacetate} + \text{NADH}_2 \qquad (1)$$

is very low, *viz.* $K = 2.3 \times 10^{-5}$ (Stern & Ochoa, 1952), the oxidation of malate to oxaloacetate can proceed only when the latter is further metabolized. It can be therefore concluded that in lamprey somatic muscle mitochondria an active metabolic removal of oxaloacetate is operating. Three possibilities can be considered: (a) formation of citrate with acetyl-CoA derived from the oxidation of pyruvate formed from malate by the malic enzyme (EC 1.1.1.38 - 40), (b) formation of citrate with acetyl-CoA derived from the oxidation of endogenous fatty acids, and (c) decarboxylation of oxaloacetate to pyruvate. Malic enzyme is very active in lamprey muscle mitochondria (Savina & A. B. Wojtczak, unpublished) and therefore the reaction (a) is possible. However, it does not seem to play the major role in removal of oxaloacetate. This is indicated by ADP/O ratio which is only slightly below 3 when malate *plus* pyruvate are oxidized, but is 2.4 with malate alone (Table 2). The theoretical

value for the ADP/O ratio for fatty acid oxidation is 2.5 (Skrede & Bremer, 1965) and therefore the reaction (b) is most likely to be the main contribution for the removal of oxaloacetate in lamprey muscle mitochondria. The possibility (c) can be excluded since no oxaloacetate decarboxylase (EC 4.1.1.3) could be found in these mitochondria (Savina & A.B. Wojtczak, unpublished).

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MITOCHONDRIA MINOGA (*LAMPETRA FLUVIATILIS*). OKSYDACYJNA FOSFORYLACJA I PROCESY POKREWNE

Streszczenie

1. Wysoką wydajność oksydacyjnej fosforylacji i dobrą kontrolę oddechową w mitochondriach wątroby, serca i mięśni somatycznych minoga (*Lampetra fluviatilis*) uzyskano, gdy organelle te izolowano w złożonym środowisku sacharozowym zawierającym EDTA, heparynę i amid kwasu nikotynowego. Dalszą poprawę sprzężenia mitochondriów obserwowano po dodaniu albuminy surowiczej do środowiska inkubacyjnego.

2. Mitochondria minoga zawierają 4 - 6 nmoli nukleotydów adeninowych na mg białka. Translokację tych nukleotydów przez błonę mitochondrialną stymuluje dodatek albuminy surowiczej.

3. Fosfolipidy mitochondriów minoga zawierają znaczny procent (64 - 72%) wielonienasyconych kwasów tłuszczowych.

4. W pracy przedstawiono także zdjęcia z mikroskopu elektronowego mitochondriów wątroby, serca i mięśni somatycznych minoga.

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ISOLATION, PURIFICATION AND PROPERTIES OF A FACTOR FROM RHEUMATOID SYNOVIAL FLUID ACTIVATING THE LATENT FORMS OF COLLAGENOLYTIC ENZYMES

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1. An activator catalysing specifically conversion of latent forms of human leucocyte collagenase and gelatin-specific protease into the active forms, has been isolated from rheumatoid synovial fluid and purified 55-fold with a yield of 16%.

2. Molecular weight of the activator is about 35 000.

3. The activator is thermolabile, and is irreversibly inactivated at pH below 5.5 or in the presence of low concentrations of trypsin or papain; it is resistant to the action of lysozyme, hyaluronidase, diisopropylfluorophosphate, soybean trypsin inhibitor, *p*-chloromercuribenzoate, iodoacetamide and dithiothreitol.

4. The activator did not show any activity towards collagen, gelatin, casein, haemoglobin, histones, elastin or *p*-phenylazobenzyloxycarbonyl-peptide.

Harper *et al.* (1971) and Harper & Gross (1972) have demonstrated that tadpole tailfin explants in tissue cultures produce, in addition to collagenase and procollagenase, an activator converting, by limited proteolysis, procollagenase into collagenase. Vaes (1972a) reported that activation of procollagenase in a culture of mouse bone explants by purified lysosomes or trypsin was due to the conversion of the latent form of the activator to its active form. The procollagenase in human leucocytes can be activated either by rheumatoid synovial fluid (Kruze & Wojtecka, 1972) or by trypsin (Oronsky *et al.*, 1973). Rheumatoid synovial fluid or trypsin and to some extent chymotrypsin activate also the latent form of gelatin-specific protease from human leucocytes (Sopata & Dancewicz, 1974).

Since activation of latent forms of collagenolytic enzymes seems to be of importance for collagenolysis in rheumatoid arthritis, we have isolated and characterized the activator present in rheumatoid synovial fluid.

MATERIALS AND METHODS

Synovial fluid. Synovial fluid was aspirated from knee joints of patients with rheumatoid arthritis; it was centrifuged for 30 min at 10 000 *g* and kept frozen until assayed.

Isolation of latent forms of collagenase and gelatin-specific protease from leucocytes. Leucocytes were isolated and homogenate prepared as described by Kruze & Wojtecka (1972) except that a buffer of a lower ionic strength: 0.02 M-Tris-HCl, pH 8.6, containing 0.002 M-$CaCl_2$, was used. The homogenate was separated on DEAE-Sephadex A-50 column in 0.02 M-Tris-HCl buffer, pH 8.6, containing 0.002 M-$CaCl_2$. Latent forms of collagenase and gelatin-specific protease were eluted using a linear gradient from 0 to 0.4 M-NaCl. Two peaks, the first eluted between 0.06 and 0.12 M-NaCl and the second between 0.17 and 0.28 M-NaCl represented the latent forms of collagenase and gelatin-specific protease, respectively. The latter was further purified by gel filtration on Sephadex G-200 column equilibrated with 0.01 M-Tris-HCl buffer, pH 7.5, containing 0.2 M-NaCl and 0.005 M-$CaCl_2$ (Tris--NaCl-$CaCl_2$ buffer).

Collagen and gelatin. Acid-soluble calf skin collagen was isolated and purified by the method of Kang *et al.* (1966). For assays an about 0.2% collagen solution obtained by dissolving lyophilized collagen in cold 0.05% acetic acid, was dialysed against 0.05 M-Tris-HCl buffer, pH 7.5, containing 0.2 M-NaCl and 0.005 M-$CaCl_2$ and then centrifuged at 10 000 *g* for 1 h.The gelatin was prepared from this collagen solution by thermal denaturation at 45°C for 15 min.

Assay of the activator. For quantitative measurements of the activator activity the following assay system was used: latent form of gelatin-specific protease (15 µg of protein) and an appropriate amount of activator were mixed and incubated for 30 min at 37°C. Thereafter 1 ml of 0.2% solution of thermally denatured collagen (gelatin) and Tris-NaCl-$CaCl_2$ buffer were added to a final volume of 2 ml. Incubation was carried out for 16 h at 37°C. The control system contained no activator. The activity of the activator was defined as the net increase in gelatinolytic activity.

The action of the activator on the latent leucocyte collagenase was estimated: (1) by measuring the net increase in collagenolytic activity of the system containing reconstituted collagen fibrils, latent collagenase and activator, incubated, without preincubation, for 16 h at 37°C in Tris-NaCl-$CaCl_2$ buffer; (2) by measuring the changes in viscosity of collagen solution containing the latent form of leucocyte collagenase with or without activator added to the system. The measurements of viscosity were carried out at 25°C using an Ostwald type viscometer.

Collagenolytic activity was measured by determination of hydroxyproline-containing peptides released from trypsin-resistant, reconstituted collagen fibrils, as described previously (Kruze & Wojtecka, 1972).

Gelatinolytic activity. Degradation of gelatin by gelatin-specific protease from leucocytes was determined by measuring the content of hydroxyproline in degradation products soluble in cold 15% trichloroacetic acid after incubation of the enzyme with gelatin at 37°C for 16 h as described by Sopata & Dancewicz (1974).

Proteolysis of the activator. The activator (15 μg of protein) was preincubated for 4 h at 37°C with trypsin (5 μg) or elastase II (5 μg) and for 30 min at 37°C with papain (3 μg) or lysozyme (50 μg) in Tris-NaCl-$CaCl_2$ buffer in a final volume of 0.5 ml. For preincubation with papain, 0.005 M-cysteine was included. Prior to the assay of the activator activity, trypsin and elastase II were inhibited by addition of diisopropylfluorophosphate (DFP) to a final concentration of 1 mM.

Molecular weight estimation. The apparent molecular weight of the activator was estimated by gel filtration on a column of Sephadex G-100 (1.6 × 75 cm). For elution Tris-NaCl-$CaCl_2$ buffer at a flow rate of 20 ml/h was used. The column was calibrated using the following standard proteins: bovine serum albumin, egg albumin, α-chymotrypsinogen A, cytochrome *c*.

Other methods. Proteolytic activity of the activator was assayed using casein (Kunitz, 1947) and urea-denatured haemoglobin (Anson, 1939) as substrates. Proteolytic activity toward azocoll was assayed as follows: 15 mg of azocoll, 0.5 ml of the activator (25 μg of protein) and 2 ml of Tris-NaCl-$CaCl_2$ buffer were incubated for 16 h at 37°C, then the mixture was filtered and the amount of dye released was measured spectrophotometrically at 520 nm. The activity toward histones was determined by the method of Davies *et al.* (1971). Elastinolytic activity was measured using the method of Banga & Ardelt (1967) except that the buffer used was 0.2 M--Tris-HCl, pH 8.8, and the time of incubation was 15 h. Assay for collagen-peptidase with 4-phenylazobenzyloxy-carbonyl-L-prolyl-L-leucyl-glycyl-L-propyl-D-arginine (PZ-peptide) as a substrate was performed according to Wünsch & Heidrich (1963) except that the buffer used was Tris-NaCl-$CaCl_2$, pH 7.5. Tryptic and chymotryptic activities were assayed as described by Schwert & Takenaka (1955). *N*-α-Benzoyl--L-arginine ethyl ester (BAEE) and *N*-acetyl-L-tyrosine ethyl ester (ATEE) were used, respectively, as the substrates. Hydroxyproline was assayed by the method of Stegemann & Stalder (1967) and protein by the method of Lowry *et al.* (1951). Polyacrylamide disc gel electrophoresis was carried out according to the method of Davis (1964). The gels were stained with Coomassie Brilliant Blue R-250.

Special reagents. Bovine testes hyaluronidase (Reanal, Budapest, Hungary); dithiothreitol, iodoacetamide, trypsin ex hog pancreas, *N*-acetyl-L-tyrosine ethyl ester and Tris (Koch-Light, Colnbrook, Bucks, England); soybean trypsin inhibitor, lysozyme from egg white, *N*-α-benzoyl-L-arginine ethyl ester (B.D.H. Laboratory Chemicals Division, England); azocoll (Calbiochem, Los Angeles, Calif., U.S.A.); α-chymotrypsinogen A, cytochrome *c* electrophoretically homogeneous (Fluka A.G., Buchs, Switzerland), bovine albumin (Sigma Chemical Comp., St. Louis, Mo., U.S.A.); Sephadex G-75, G-100, G-150 and DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Uppsala, Sweden); Coomassie Brilliant Blue R-250 (Serva, Feinbiochemica, Heidelberg, G.F.R.); *p*-chloromercuribenzoate (Chemapol, Praha,

Czechoslovakia); elastase II (Ardelt, 1974) was kindly offered by Dr. W. Ardelt from this laboratory. Traskolan (Trasylol) (Polfa, Warsaw, Poland); other reagents were commercial products of analytical grade.

RESULTS

Isolation of activator from rheumatoid synovial fluid. The purification procedure is summarized in Table 1. The activity of activator in the course of purification was assayed using the latent form of gelatin-specific protease as a substrate. Pooled synovial fluid was pretreated with hyaluronidase (110 ml of synovial fluid and 29 mg of hyaluronidase dissolved in 5 ml of 0.05 M-Tris-HCl buffer, pH 7.5, containing 0.005 M-$CaCl_2$ was incubated for 16 h at room temperature), and then dialysed against Tris-NaCl-$CaCl_2$ buffer; the solution was centrifuged at 10 000 *g* for 10 min.

Table 1

Purification of the activator from rheumatoid synovial fluid

Specific activity refers to micrograms of hydroxyproline solubilized from gelatin after activation of latent form of gelatin-specific protease by 1 mg of activator.

Purification step	Total protein (mg)	Spec. act. (μg Hypro/ /mg)	Total act. (μg Hypro)	Yield (%)
Synovial fluid	450	150	67 500	100
$(NH_4)_2SO_4$ ppt. (0 - 0.5 sat.)	235	300	70 500	104
Sephadex G-150	49	1300	63 700	94
DEAE-Sephadex A-50	33	2000	66 000	97
Sephadex G-100	7	3800	26 000	38
Sephadex G-75	1.4	8000	11 200	16

Solid ammonium sulphate was added to the supernatant to 0.5 saturation and the suspension was allowed to stand for 1 h in an ice-bath; the precipitate collected by centrifugation at 10 000 *g* for 10 min was dissolved in a small volume of Tris-NaCl--$CaCl_2$ buffer, dialysed against 0.1 M-Tris-HCl buffer, pH 7.5, containing 1 M-NaCl and 0.005 M-$CaCl_2$, and filtered through a column of Sephadex G-150 (2.4 × 77 cm) equilibrated with the same buffer. The effluent fractions which contained the activator were pooled, dialysed against 0.02 M-Tris-HCl buffer, pH 7.6, with 0.005 M--$CaCl_2$ and further purified by ion-exchange chromatography on a column of DEAE--Sephadex A-50 (1.4 × 16 cm) equilibrated with the same buffer. Elution was performed by applying a continuous gradient of NaCl from 0 to 0.5 M added to the buffer. For further purification, the activator was separated on Sephadex G-100 column (1.6 × 75 cm), followed by filtration on a column of Sephadex G-75 (1.9 × × 75 cm). The effluent fractions containing the activator were pooled and stored at −20°C. All operations were carried out at 4°C unless stated otherwise. The elution pattern obtained on Sephadex G-150 filtration revealed a peak of the activator superimposed partially on the synovial fluid collagenase (Fig. 1). Separation of the

two activities was achieved by chromatography on DEAE-Sephadex A-50. Two peaks, the first eluted between 0.07 - 0.13 and the second between 0.15 - 0.25 M--NaCl represented, respectively, the synovial fluid collagenase and the activator (Fig. 2). Further purification of the activator by gel filtration on Sephadex G-100 and Sephadex G-75 is illustrated in Figs. 3 and 4. From the latter column, the activator emerged as a single peak shortly after void volume of the column. The over--all purification was about 55-fold with a yield of 16% (Table 1).

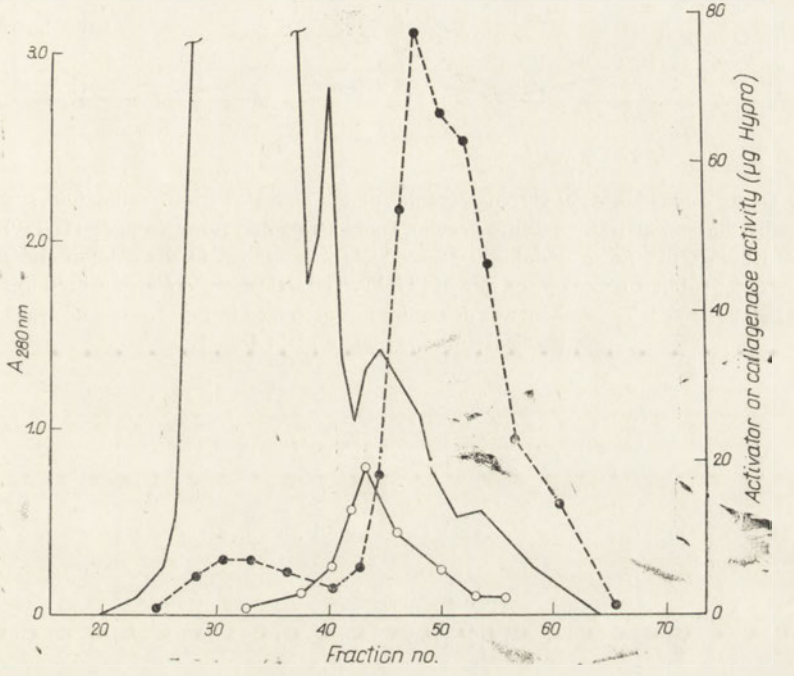

Fig. 1. Sephadex G-150 column chromatography of the activator from rheumatoid synovial fluid. Protein, 230 mg (redissolved $(NH_4)_2SO_4$ ppt.) was applied to the column and eluted with 0.1 M-Tris--HCl buffer, pH 7.5, containing 0.005 M-$CaCl_2$ and 1 M-NaCl, at a flow rate of 12 ml/h. Effluent fractions of 4 ml were collected and the activator and collagenase activities were determined as described in Materials and Methods. The activity of activator, assayed as net increase in activity of gelatin-specific protease (●), and the activity of synovial fluid collagenase (○) are expressed in micrograms of hydroxyproline solubilized from the respective substrate; ——, $A_{280\,nm}$.

Disc electrophoresis on polyacrylamide gel showed that this final preparation of the activator was still not homogeneous (Fig. 5). Only one of the three protein bands seen in the gel showed the ability to activate the latent forms of collagenase and gelatin-specific protease. This was demonstrated by analysing the activity of the eluates from the gel slices containing the separated protein bands.

Molecular weight of the activator was calculated from the position of the peak of activity in effluents from the column of Sephadex G-100. The column was calibrated with bovine serum albumin (mol. wt. 68 000), egg albumin (mol. wt. 44 000),

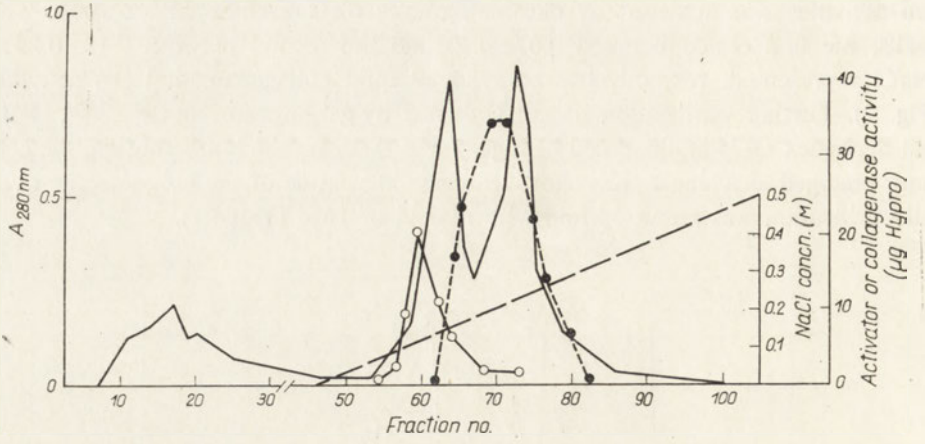

Fig. 2. DEAE-Sephadex A-50 chromatography of the activator from rheumatoid synovial fluid. A partially purified activator (40 mg protein, fractions 45 - 60 from Sephadex G-150) in 0.02 M--Tris-HCl buffer, pH 7.6, containing 0.005 M-$CaCl_2$ was applied to the column and the effluent fractions of 3.5 ml were collected at a rate of 12 ml/h. The activator was eluted using a linear gradient from 0 to 0.5 M-NaCl. The activity was determined as described in Fig. 1. — — —, NaCl concentration gradient; other symbols as in Fig. 1.

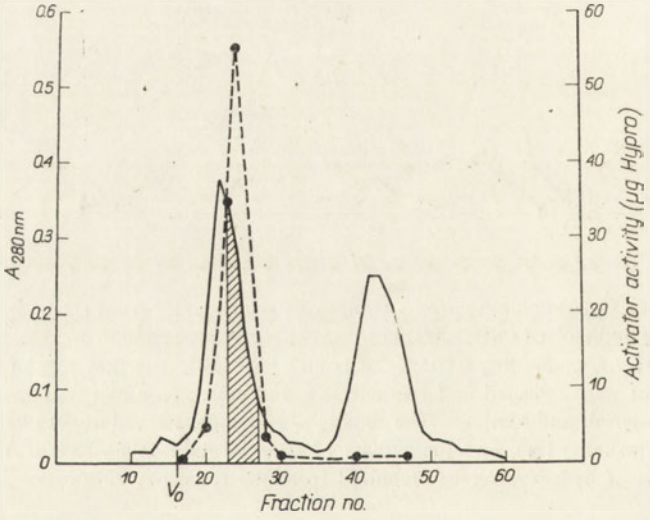

Fig. 3. Sephadex G-100 chromatography of the activator. The activator separated by chromatography on DEAE-Sephadex (7 mg, fractions 66 - 80) was applied to the column and eluted with Tris-NaCl-$CaCl_2$ buffer at a flow rate of 18 ml/h; fractions of 3.4 ml were collected and assayed for the activation of gelatinolytic activity as described in Materials and Methods. The activity (●) is expressed in micrograms of hydroxyproline solubilized; ——. $A_{280\,nm}$.

α-chymotrypsinogen A (mol. wt. 25 000) and cytochrome *c* (mol. wt. 1 1700). The approximate molecular weight of the activator evaluated by this method was 35 000 (Fig. 6).

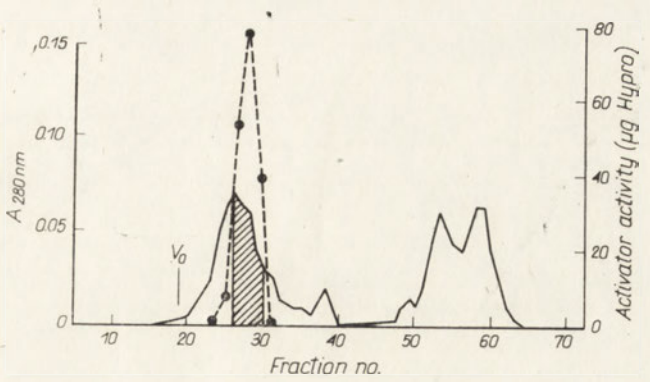

Fig. 4. Sephadex G-75 chromatography of the activator. The activator separated by chromatography on Sephadex G-100, 2 mg, was applied to the column; effluent fractions of 3.2 ml were collected at a rate of 18 ml/h and assayed for activation of gelatinolytic activity (●) as described in Materials and Methods and Fig. 1. The activity is expressed in micrograms of solubilized hydroxyproline. ——, $A_{280\,nm}$.

Properties of the activator. The isolated purified activator activated about 12 times the latent form of leucocyte collagenase and about 90 times that of gelatin-specific protease (Table 2). The activator alone had no activity toward collagen or gelatin. The viscosity measurements demonstrated that activation of latent collagenase by the activator resulted in a net 30% decrease in viscosity on 6 h incubation at 25°C (Fig. 7). The pattern of collagen degradation products resulting from the action of activated collagenase (Fig. 8) was similar to that generally obtainable with tissue collagenases (Harris *et al.*, 1969; Kruze & Wojtecka, 1972).

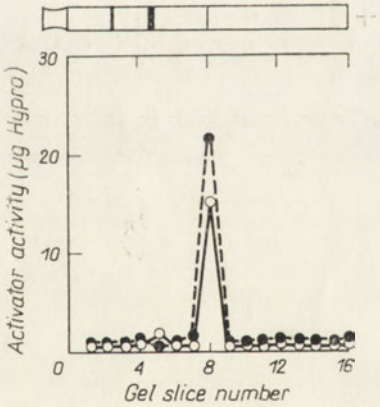

Fig. 5. Polyacrylamide disc gel electrophoresis of the 55-fold purified activator from rheumatoid synovial fluid. To the gels, 60 µg of the activator preparation was applied. The electrophoresis was carried out at 5 mA per tube. After electrophoresis one gel was stained with Coomassie Brilliant Blue and another cut into transverse discs. Each disc was eluted with 0.5 ml of Tris-NaCl-$CaCl_2$ buffer and the eluate assayed for the activator activity using the latent forms of collagenase (○) and gelatin-specific protease (●).

As can be seen from Fig. 9A, conversion of the latent form of gelatin-specific protease to the active enzyme was linearly dependent on the activator concentration up to the conversion of about 80% of the latent form present in the system. All determinations of the activator activity were made at concentrations fulfilling kinetic linearity. The maximum activation was attained after 30 min incubation of the

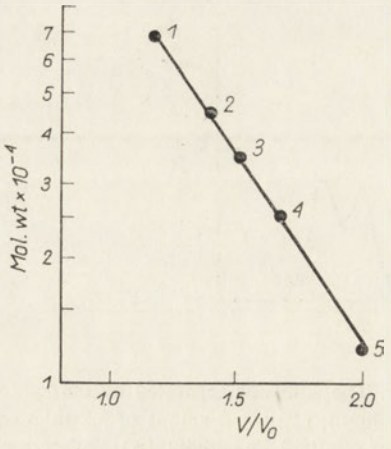

Fig. 6

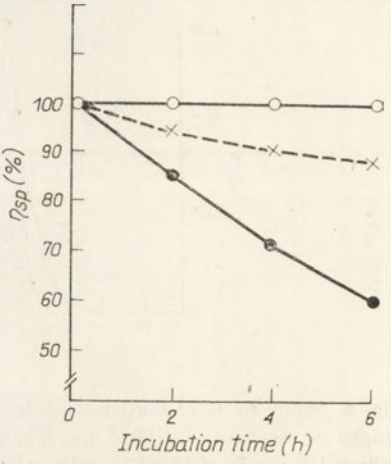

Fig. 7

Fig. 6. Determination of molecular weight of the activator from rheumatoid synovial fluid. Sephadex G-100 column at pH 7.5 calibrated with standard proteins was used. Effluent fractions of 3.4 ml were collected at a rate of 18 ml/h. Protein concentration was determined by measuring the absorbance at 280 nm. *1*, Bovine serum albumin; *2*, egg albumin; *3*, activator from synovial fluid; *4*, α-chymotrypsinogen A; *5*, cytochrome *c*.

Fig. 7. Viscometric assay of collagenolytic activity in the presence of the activator from rheumatoid synovial fluid. Latent collagenase (0.75 ml, 1 mg of protein), 1.25 ml of activator (150 μg of protein), 1 ml of 0.2% collagen solution and 0.05 M-Tris-HCl buffer, pH 7.5, containing 0.005 M-$CaCl_2$ and 0.3 M-NaCl in a final volume of 3 ml, were mixed in Ostwald's viscometer. Measurements were performed at 25°C. ○, Activator; ×, latent form of collagenase; ●, latent form of collagenase *plus* activator.

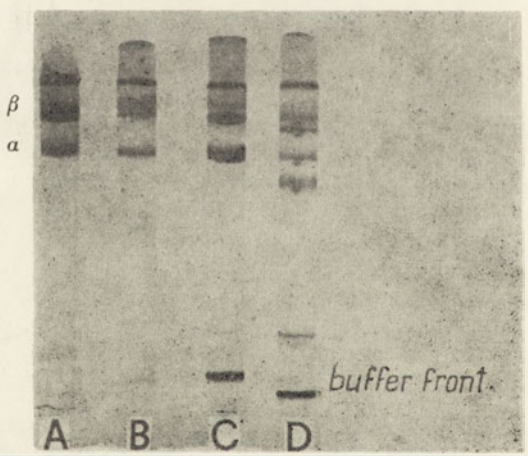

Fig. 8. Acrylamide-gel electrophoresis of: *A*, collagen; *B*, collagen treated with activator; *C*, collagen treated with latent leucocyte collagenase; *D*, collagen treated with latent leucocyte collagenase and activator. α refers to the single polypeptide chain, β to the cross-linked dimers of the α chains. Electrophoresis was preceded by thermal denaturation of the protein constituents of the incubation mixture used in the viscosity assay.

Table 2

Effect of the activator from rheumatoid synovial fluid on the latent forms of leucocyte collagenase and gelatin-specific protease

The incubation system consisted of: activator (0.1 ml, 20 μg of protein); latent form of collagenase (0.1 ml, 200 μg of protein) or gelatin-specific protease (0.05 ml, 15 μg of protein); substrate for active enzyme (0.2% collagen, 1 ml, or 0.2% gelatin, 1 ml), and buffer added to a final volume of 2 ml. Collagenolytic and gelatinolytic activities were determined as described in Methods and expressed in micrograms of hydroxyproline solubilized, respectively, from collagen or gelatin.

Incubation system	Activity	
	collagenolytic	gelatinolytic
Complete	60.0	90.0
minus activator	4.8	1.2
minus latent collagenase	0.0	—
minus latent gelatin-specific protease	—	0.0

latent form of gelatin-specific protease with the activator (Fig. 9B). Longer incubation, up to 3 h, had no effect on the reached level of the activity.

The effect of pH on stability of the activator is presented in Fig. 10. As it can be seen, the activator is stable between pH 5.5 and 8.0. A 30 min incubation at pH below 5.0 or above 9.0 caused a marked, irreversible loss of the activating property. At pH 7.5 the activator can be stored at −20°C for a month without any loss of the activity. On the other hand, heating at pH 7.5 for 5 min at 100°C or 20 min

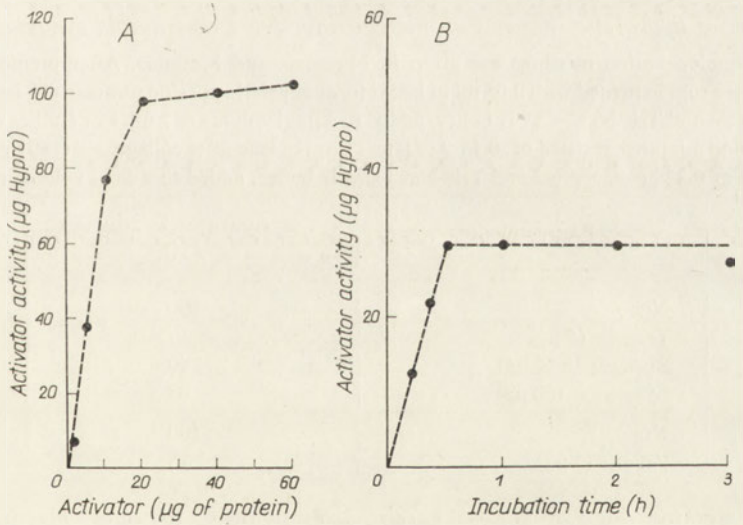

Fig. 9. Activation of latent form of gelatin-specific protease by synovial fluid activator: *A*, as a function of activator concentration, and *B*, as a function of time. Conditions of the enzymic assay for *A* were as described in Methods; for *B* the incubation mixture consisted of 0.03 ml of latent form of gelatin-specific protease (15 μg of protein) and 0.2 ml of activator (30 μg of protein); the reactants were mixed and preincubated at 37°C during the indicated time, then 1 ml of gelatin was added and Tris-NaCl-$CaCl_2$ buffer to a final volume of 2 ml. This mixture was incubated for 4 h at 37°C and the activator activity was assayed.

at 65°C decreased the activity by 25%, whereas heating for 10 min at 100°C destroyed the activity completely.

The activator was not affected by 1 or 2 mM-DFP, nor by soybean trypsin inhibitor (0.1 or 0.2 mg/ml). Dithiothreitol (10 mM) and traskolan (250 or 2500 inactivator kallikrein units/ml) were also practically ineffective, whereas PCMB (1 mM) and iodoacetamide (1 mM) inhibited the activator by about 25%.

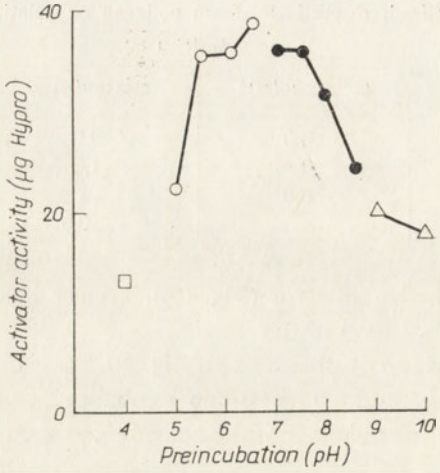

Fig. 10. Effect of pH on the stability of the activator from rheumatoid synovial fluid. The activator was preincubated for 30 min at 37°C in the presence of the following buffers: 0.23 M-glycine-CH_3COOH, pH 4.0 (□); 0.2 M-Tris-maleate, pH range 5.0 - 7.0 (○); 0.2 M-Tris-HCl, pH range 7.5 - 8.5 (●); 0.2 M-glycine-NaOH, pH 9.0 and 10.0 (△). At the end of preincubation the pH was adjusted to 7.5 by the addition of 1.4 ml of 0.2 M-Tris-HCl buffer, pH 7.5, containing 0.005 M-$CaCl_2$, to 0.1 ml of preincubation mixture. The activator activity was assayed as described in Materials and Methods.

Table 3

Effect of hydrolytic enzymes on the activator from rheumatoid synovial fluid

Details of preincubation mixtures are given in Materials and Methods. After preincubation the mixtures were supplemented with 0.05 ml of latent form of gelatin-specific protease (15 μg of protein), 1 ml of gelatin and Tris-NaCl-$CaCl_2$ buffer, added to a final volume of 2 ml. For studies with papain, the incubation mixture consisted of: 0.1 ml of latent form of leucocyte collagenase (160 μg of protein), 0.5 ml of 0.2% collagen gel and Tris-NaCl-$CaCl_2$ buffer, added to a final volume of 2 ml.

Pretreatment	Activator activity (%)
None	100
Trypsin (5 μg)	7
Elastase II (5 μg)	40
Lysozyme (50 μg)	85
None	100
Papain (3 μg)	15

The activator did not act on casein, haemoglobin, histones, elastin, azocoll, PZ-peptide, ATEE or BAEE, and it was unable to activate chymotrypsinogen. On the other hand, trypsin and papain destroyed the activator activity, while lysozyme had no effect even at relatively high concentration. The activator was also partially destroyed by elastase II (Table 3). It should be noted that the activator was resistant to the action of hyaluronidase which was used during isolation of the activator from the synovial fluid.

DISCUSSION

The ability of rheumatoid synovial fluid to activate human leucocyte procollagenase has been first reported by Kruze & Wojtecka (1972). Sopata & Dancewicz (1974) have shown that rheumatoid synovial fluid activates also a latent form of gelatin-specific protease, the enzyme accompanying collagenase in human leucocytes. The results described in this paper demonstrate that a property of rheumatoid synovial fluid to convert both these latent forms into their respective enzymes may be ascribed to a specific protein.

The isolation and purification procedures applied in this work resulted in obtaining an active preparation of a protein which on Sephadex G-75 emerged as a single peak of mol. wt. 35 000. The ability of this protein to activate the latent form of gelatin-specific protease is 55 times higher than that of the original rheumatoid synovial fluid. However, on polyacrylamide-gel electrophoresis the preparation is still inhomogeneous.

The latent forms of collagenase and gelatin-specific protease are the only substrates of the activator. It does not hydrolyse collagen, gelatin, PZ-peptide or a few other natural and synthetic substrates. Thus it seems reasonable to consider the isolated protein as an activator similar in its function to the activators of procollagenase detected in tissue cultures (Harper *et al.*, 1971; Vaes, 1972a). The question of identity of these activators remains open until isolation and better characterization of the activators from the explants.

Vaes (1972b) regards the activator present in the culture medium of mouse bone explants as a specific protease, and Harper *et al.* (1971) have demonstrated that activation of procollagenase of the tissue explants is associated with a decrease in molecular weight of this protein. We have no conclusive evidence that the activator from synovial fluid is a specific protease as we have not been able to detect a scission product of the polypeptide chain during the activation process, although the properties of the activator are consistent with its being a protease.

The origin of the activator in the rheumatoid synovial fluid has not been unequivocally established. We have demonstrated that tissue cultures of synovium explants from rheumatoid arthritis patients produce an activator which converts leucocyte procollagenase into collagenase and which seems to be identical with the activator isolated from rheumatoid synovial fluid (Wize *et al.*, 1975). The possibility that the activator could be produced directly from an unknown inactive protein present in the synovial fluid cannot be excluded. The existence of a latent form of an activator in bone tissue culture has been suggested by Vaes (1972a).

The fact that synovial fluids from osteoarthritic patients are unable to activate procollagenase from human leucocytes (Oronsky *et al.*, 1973) suggests that production of the activator may be attributed to rheumatoid arthritis only. If this were the case, the presence of the activator in the synovial fluid could be utilized for diagnosis of this disease.

Our results allow us to regard the activator as a key agent in the enhancement of extracellular degradation of collagen in rheumatoid arthritis.

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IZOLACJA, OCZYSZCZANIE I WŁASNOŚCI CZYNNIKA Z REUMATOIDALNEGO PŁYNU STAWOWEGO AKTYWUJĄCEGO NIECZYNNE FORMY ENZYMÓW KOLAGENOLITYCZNYCH

Streszczenie

1. Z płynu stawowego wyizolowano czynnik aktywujący nieczynne formy kolagenazy i obojętnej proteazy leukocytów ludzkich. Aktywator ten oczyszczono ok. 55-krotnie ze stratą 84% aktywności.

2. Ciężar cząsteczkowy aktywatora określono na 35 000.

3. Aktywator jest ciepłochwiejny i nieodwracalnie hamowany przez niskie stężenia trypsyny i papainy oraz pH poniżej 5.5. Lizozym, hialuronidaza, dwuizopropylofluorofosforan, *p*-chlororęciobenzoesan, jodoacetamid i dwutiotreitol nie wpływają na aktywność aktywatora.

4. Aktywator nie wykazuje aktywności wobec kolagenu, żelatyny, kazeiny, hemoglobiny, histonów, elastyny i *p*-fenyloazobenzyloksykarbonylo-peptydu.

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ON THE ARRANGEMENT OF HISTONES IN CHROMATIN*

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It was found that nucleoprotein particles formed after DNase I action on calf thymus chromatin contain single-stranded DNA fragments, associated with histones only by ionic linkages. These results suggest that histones in chromatin are bound ionically only to one polynucleotide strand of double-helical DNA, protecting it against nucleolytic attack.

Several lines of evidence point to the existence of a regularity of chromatin substructure. Chromatin can be readily depolymerized by nucleases into particles containing DNA fragments homogeneous in size (Clark & Felsenfeld, 1971; Kaliński *et al.*, 1972). Recently Shaw *et al.* (1974) have found that digestion of chromatin by staphylococcal nuclease produces nucleoprotein particles of 11.2 S with double-stranded DNA of about 0.8×10^5 daltons. On the other hand, Oliver & Chalkley (1974) have shown that the major macromolecular product formed on pancreatic nuclease (DNase I) action is a positively charged complex containing single-stranded DNA associated with histones. The authors proposed a new model for nucleohistone primary structure, the main feature of which is "the asymmetric distribution of histones about the two strands of DNA, lying in both the major and minor grooves, thus sterically protecting one strand against nucleolytic attack... while leaving the other strand more available".

Kaliński *et al.* (1972) have shown that the extent of degradation of DNA in chromatin and the size of DNase I resistant fragments of DNA are influenced in varying degrees by particular histone fractions. Digestion by DNase after removal of histone *f1* led to formation of fragments about ten times smaller than those obtained on digestion of whole chromatin.

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In this paper we present further data concerning the properties of DNase I resistant fragments of calf thymus chromatin, and propose the mode of arrangement of histones on chromatin DNA.

MATERIALS AND METHODS

Chromatin was prepared from calf thymus according to Marushige & Bonner (1966) except that ultracentrifugation through 1.7 M-sucrose was omitted.

DNase-resistant fragments of chromatin. Chromatin was digested with pancreatic deoxyribonuclease (DNase I) under conditions described by Kaliński *et al.* (1972). The incubation mixture contained: 10 mM-Tris-HCl, pH 8.0, 3 mM-$MgCl_2$, 60 μg/ml of DNase I, and 20 E_{260} units of chromatin. The digestion was carried out at 37°C for 30 min, then the mixture was cooled to 0°C, centrifuged at 12 000 *g* for 15 min, and the sediment washed five times with 10 mM-Tris-HCl buffer, pH 8.0. The obtained sediment was used as the DNase-resistant fragments of chromatin.

Extraction of histones from chromatin with 0.6 M-NaCl or 0.6 M-NaCl - 0.15% Triton X-100 was performed as described by Toczko & Kaliński (1974).

Extraction of histones from DNase-resistant fragments. To DNase-resistant fragments containing DNA equivalent to 50 E_{260} units, was added 25 ml of 0.6 M-NaCl, stirred for 1 h at 0°C and centrifuged at 3500 *g* for 15 min. Then 10 ml of the solution containing 2 E_{260} units/ml was applied to Sepharose 6B column to separate histones dissociated from DNase-resistant fragments (Fig. 1).

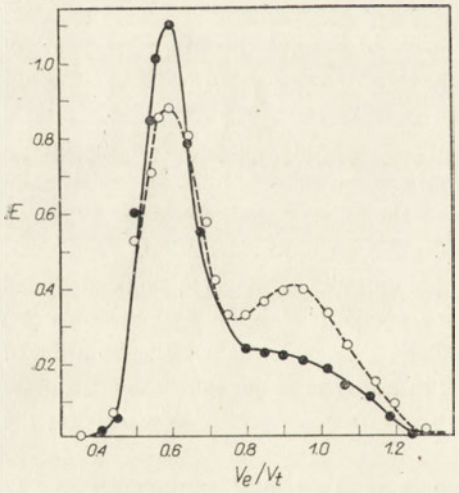

Fig. 1. Elution profiles from Sepharose 6B column of DNase-resistant fragments of chromatin DNA treated with 0.6 M-NaCl. The preparation was applied to the column (30 × 1.6 cm, V_t 60 ml) equilibrated and eluted with 0.6 M-NaCl. (●), $E_{260\,nm}$; (○), $E_{230\,nm}$.

Isolation of DNA from chromatin and DNase-resistant fragments. This was performed according to Stern (1968) as described by Kaliński *et al.* (1972).

Melting curves. The changes in absorbance were followed in 0.25 mM-EDTA, pH 7.0, at 260 nm, using Zeiss VSU-2P (Jena, G.D.R.) spectrophotometer, the increase in temperature being 1°C/min.

Determination of DNA. The concentration of DNA was calculated from the value of E_{260} assuming $E^{1\%}_{1\,cm}$ at 260 nm to be 210.

Determination of histones was carried out after Lowry *et al.* (1951) with total histones from calf thymus as standard.

Electrophoresis. Polyacrylamide-gel electrophoresis of histones was performed by the method of Panyim & Chalkley (1969). Gels were scanned using an ERJ 65 densitograph (Carl Zeiss, Jena, G.D.R.).

Reagents. Pancreatic deoxyribonuclease (DNase I) was purchased from Worthington Biochemical Corporation (Freehold, N.J., U.S.A.). Sepharose 4B and 6B were from Pharmacia (Uppsala, Sweden). Reagents for gel electrophoresis were from Serva (Heidelberg, G.F.R.).

RESULTS AND DISCUSSION

In the previous paper (Kaliński *et al.*, 1972) it has been demonstrated that about a half of DNA in calf thymus chromatin is readily degraded by pancreatic deoxyribonuclease (DNase I) to acid-soluble oligonucleotides while the other half remains in the form of DNase-resistant fragments associated with proteins.

In the present experiments it was found that after digestion by DNase of 49 - 52% of the DNA present in chromatin (Table 1), the weight ratio of histone to DNA in the DNase-resistant fragments was about 2.0, i.e. almost twice as high as in the case of intact chromatin. Electrophoretic analysis showed that these particles contained all five histone fractions in ratios similar to those observed in chromatin (see Fig. 3).

Table 1

DNA and histone weight ratios in chromatin and DNase I resistant fragments

DNase-resistant fragments were prepared from calf thymus chromatin after DNase I digestion (60 μg/ml) at 37°C for 30 min as described by Kaliński *et al.* (1972). For details see also Materials and Methods.

Preparation	Hydrolysis of DNA in chromatin (%)	Histone: DNA weight ratio
Chromatin	0	1.11
		1.20
DNase-resistant fragments	49	1.98
	52	1.96
	51	1.87
	50	2.00

The melting profile of DNA isolated from chromatin was characteristic of the double-helical structure, with a hyperchromicity of 40% (Fig. 2). On the other hand, the thermal denaturation profile of DNA prepared from DNase-resistant fragments resembled that of the single-stranded DNA, which is in agreement with

the results of Oliver & Chalkley (1974). The degradation of only a single strand of DNA assumes that the accessibility of DNA in chromatin to DNase I digestion is determined not by the lack of histones but rather by the mode of their binding to DNA. The analysis of the nature of the interaction of particular histones with DNA indicated that histone *f1* was bound only by ionic linkages while for other histones the contribution of hydrophobic interactions was significant (Toczko & Kaliński, 1974; Kaliński & Toczko, 1974).

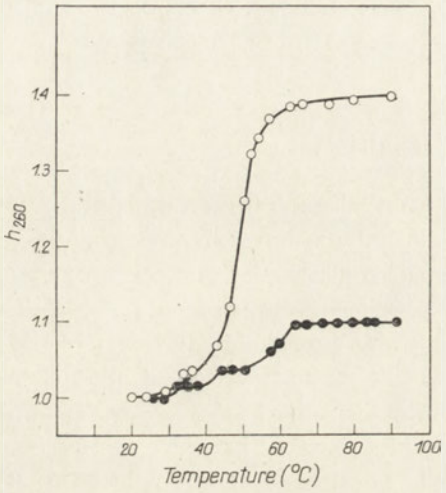

Fig. 2. Melting profiles of DNA isolated from: (○), calf thymus chromatin and (●), DNase-resistant fragments. The melting was performed in 0.25 mM-EDTA at pH 7.0. Hyperchromicity (h_{260}) was calculated by dividing the absorbance at a given temperature by the value obtained at room temperature.

Table 2

Dissociation of histones from chromatin by NaCl

The preparation of chromatin or DNase-resistant fragments were mixed for 1 h at 0°C with solutions of 0.6 M-NaCl or 0.6 M-NaCl - 0.15% Triton X-100. The dissociated histones were separated by gel chromatography on Sepharose 4B (chromatin) or Sepharose 6B (DNase-resistant fragments). The percentage of dissociated histones in particular preparations was calculated from histone: :DNA weight ratios determined before and after separation of dissociated histones.

Preparation	Dissociating agent	Dissociated histones (%)
Chromatin	0.6 M-NaCl	18
		19
		19
Chromatin	0.6 M-NaCl - 0.15% Triton X-100	95
		94
DNase-resistant fragments	0.6 M-NaCl	44
		46

In the present work, the contribution of either type of interactions between histones and DNA in DNase-resistant fragments was evaluated by determining the dissociation of histones in salt solution. In 0.6 M-NaCl, about 45% of histones dissociated (Table 2) and the electrophoretic pattern (Fig. 3C) showed the presence

of all histone fractions. Under the same conditions, from undigested chromatin 19% of histones dissociated (Table 2) and on gel electrophoresis only the histone fraction *f1* was found (Fig. 3A), whereas in 0.6 M-NaCl containing 0.15% Triton X-100, 95% of histones was dissociated. The presented data clearly indicated that the DNase-resistant fragments of chromatin lost most of the hydrophobic interactions. These results allow us to conclude that only those fragments of polynucleotide strand of DNA in chromatin are resistant to DNase I which are ionically bound by their phosphate groups to histones. Thus it appears that in a given DNA fragment histones are ionically bound to only one polynucleotide strand.

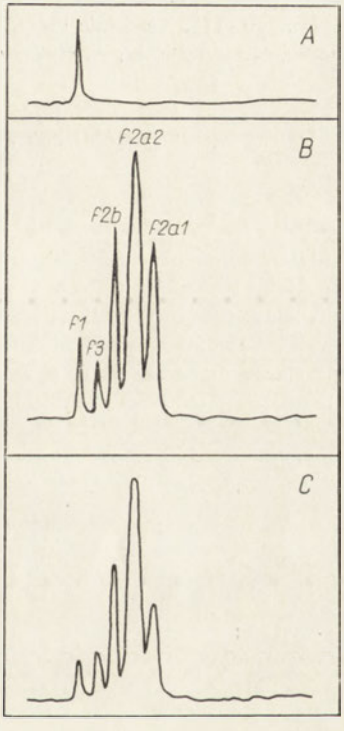

Fig. 3. Densitometer scans of electrophoretic patterns of histones dissociated from: *A*, chromatin extracted with 0.6 M-NaCl; *B*, chromatin extracted with 0.6 M-NaCl - - 0.15% Triton X-100; *C*, DNase-resistant fragments extracted with 0.6 M-NaCl.

In our previous work we have shown (Kaliński *et al.*, 1972) that DNase I resistant fragments of DNA in chromatin are homogeneous in size and rather large ($s_{20,w}$ 2.9). The size of these fragments depends strongly on the presence of histone *f1* since its dissociation from chromatin prior to digestion resulted in about tenfold decrease in the length of undigested DNA fragments. Therefore we assumed that histone *f1* is bound ionically in extended form to polynucleotide strand, the remaining four histones being ionically bound only by small fragments of their polypeptide chains. Taking into consideration that only 50% of DNA in chromatin is readily hydrolysed by DNase I and assuming that only one polynucleotide strand of double-helical DNA is degraded, it may be further concluded that calf thymus chromatin does not contain long stretches of DNA not covered by proteins, as postulated by Clark & Felsenfeld (1971).

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O ROZMIESZCZENIU HISTONÓW W CHROMATYNIE

Streszczenie

Ustalono, że cząstki nukleoproteidowe powstałe w wyniku trawienia chromatyny dezoksyrybonukleazą trzustkową (DNazą I) zawierają odcinki DNA zbudowane z pojedynczych łańcuchów polinukleotydowych. Wszystkie histony w tych cząstkach związane są z fragmentami DNA wyłącznie wiązaniami jonowymi. Wyniki te dowodzą, że histony w chromatynie na danym odcinku dwułańcuchowej cząsteczki DNA są związane jonowo tylko z jednym łańcuchem polinukleotydowym, zabezpieczając ten łańcuch przed działaniem nukleazy.

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ACYL-CoA POOL AND ACYL-CoA THIOESTERASE IN *STREPTOMYCES NOURSEI* VAR. *POLIFUNGINI****

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The size of acyl-CoA pool in *S. noursei* var. *polifungini* was found to be associated with the antibiotic-synthesizing ability, and was negatively correlated with both the thioesterase activity (acetyl-CoA hydrolase, EC 3.1.2.1) and its affinity towards acetyl-CoA and propionyl-CoA. The apparent Michaelis constants with acetyl-CoA and propionyl-CoA were 33×10^{-5} and 6.6×10^{-5} M in the low producing strain, and 8.5×10^{-4} and 1.2×10^{-4} M in the high producing strain, respectively.

Acetyl-CoA and propionyl-CoA are polymerized to form the lactone rings of macrolide antibiotics (Manwaring *et al.*, 1969) *via* malonyl-CoA and methylmalonyl--CoA (Lynen, 1967).

The results presented in this paper point to the importance of thioesterases in maintaining acetyl-CoA and propionyl-CoA pool available for the synthesis of polifungin, a poliene antibiotic of nystatin type produced by *Streptomyces noursei* var. *polifungini* (Porowska *et al.*, 1972). A preliminary account of this work has been presented at the II Int. Symposium "Recent Progress in Antibiotic Research" (Raczyńska-Bojanowska, 1974).

MATERIALS AND METHODS

Reagents. Lysozyme and Tris were products of Boehringer und Soehne (Mannheim, G.F.R.), coenzyme A was from Sigma (St. Louis, Mo., U.S.A.), and propionic anhydride from Union Chimique Belgique (Bruxelles, Belgium). Dithionitrobenzoic

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acid (DTNB) was from Calbiochem (Los Angeles, Calif., U.S.A.). Other reagents were from Biuro Odczynników Chemicznych (Gliwice, Poland). Propionyl-CoA and acetyl-CoA were synthesized by the method of Simon & Shemin (1953) from the corresponding anhydrides.

Strains and culture conditions. The low producing 820/25 and high producing R 851/26 u.v. mutants of *Streptomyces noursei* var. *polifungini* (ATTC 25581) were obtained from Mrs. D. Kotiuszko, M.Sc., of the Division of Microbiology of the Institute of Pharmaceutical Industry. The organisms were grown in submerged cultures for 96 h at 28°C on carbohydrate or lipid medium as described previously (Rafalski & Raczyńska-Bojanowska, 1972). The yield of antibiotic obtained with the high producing mutant R 851/26 was 21 200 units/ml on carbohydrate and about 25 000 units/ml on lipid medium. Mutant 820/25 produced traces of antibiotic, not detectable microbiologically, on both media applied.

Preparation of extracts. Cell extracts for the metabolite and enzyme assays were prepared as described previously (Rafalski & Raczyńska-Bojanowska, 1972).

Determination of acyl-CoA pool. The total acyl-CoA pool was estimated by measuring the extinction at 412 nm on addition of DTNB (Tubbs & Garland, 1969) to the cell extract previously hydrolysed for 15 min at 37°C in 0.1 M-NaOH to decompose thioesters. The reaction mixture contained in a total volume of 3 ml: DTNB, 25 μmoles (0.5 ml), Tris-HCl buffer, pH 8.0, 400 μmoles, and the hydrolysed extract (1 ml). The control sample contained the non-hydrolysed extract. Concentration of acyl-CoA was expressed in nmoles/g of mycelium wet weight.

Determination of the activity of acetyl-CoA and propionyl-CoA thioesterases. The enzymic activities were determined in the cell extracts dialysed on Sephadex G-25 coarse, and in the spheroplasts and the cell wall fractions separated on lysozyme treatment of the cells (Ruczaj *et al.*, 1969).

The activity of acetyl-CoA and propionyl-CoA thioesterases was measured after Srere (1969) in the extracts dialysed on Sephadex G-25, by monitoring the rate of CoASH liberation in the presence of DTNB at 412 nm. The reaction mixture contained in a total volume of 2.5 ml: Tris-HCl buffer, pH 8.0, 50 μmoles; DTNB, 5 μmoles; acetyl- or propionyl-CoA, 1 μmole; and 0.05 - 0.4 ml of the dialysed enzymic extract containing 0.25 - 2.0 mg of protein. The activity was expressed in nmoles/min/mg protein. In the control sample, acyl-CoA was omitted.

Determination of protein was performed after Lowry *et al.* (1951) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Concentration of acyl-CoA pool was considerably higher, up to 30-fold, in the 72-h cultures of the high-producing R 851/26 strain as compared with the 820/25 strain producing polifungin in trace amounts (Fig. 1). In the first two days of cultivation of the 820/25 strain acyl-CoA could not be detected or its amount was very low. The difference between the strains did not depend on the kind of nutrient medium used, and was equally pronounced when the acyl-CoA derivatives origi-

nated from lipids used as a sole carbon source or when they were synthesized from the intermediates of glycolysis in the cultures grown on carbohydrates.

The high pool of acyl-CoA in R 851/26 was accompanied by a low activity of thioesterases with acetyl-CoA and propionyl-CoA (Fig. 2). In the low producing strain these activities were from 7 to 30 times higher, with the correspondingly lower acyl-CoA pool. The activity of deacylation systems in R 851/26 strain (20 nmoles/min/mg protein) was 10^2 - 10^3 times higher than that of acetyl-CoA and propionyl-CoA carboxylases (0.01 and 0.001 nmole/min/mg protein, respectively) and was of the same order of magnitude as the activity of methylmalonyl-CoA carboxyltransferase and the enzyme system activating acetate and propionate to acetyl-CoA and propionyl-CoA (Rafalski & Raczyńska-Bojanowska, 1973; Raczyńska-Bojanowska, 1974). The obtained results point to the high specific activity of deacylation systems in *S. noursei* var. *polifungini*. In the low producing strain, thioesterase activity exceeded the above-mentioned enzymic activities about 50-fold.

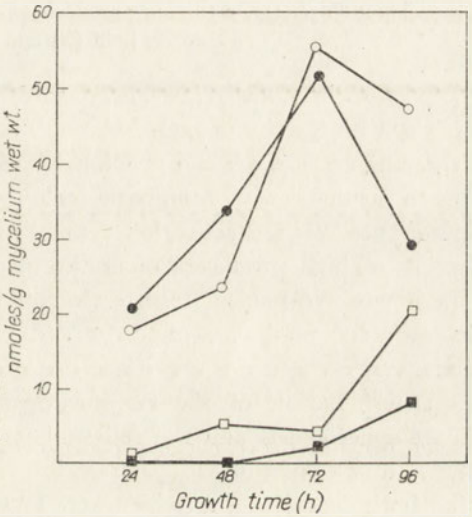

Fig. 1. The acyl-CoA pool in the high producing R 851/26 (○, ●) and low producing 820/25 (□, ■) strains of *S. noursei* var. *polifungini* grown on lipid (○, □) and carbohydrate (●, ■) media.

Not only the activity but also the affinity towards acetyl-CoA and propionyl-CoA were higher in the low producing strains: the K_m values for acetyl- and propionyl-CoA were 33×10^{-5} and 6.6×10^{-5} M in the 820/25 strain, and 8.5×10^{-4} and 1.2×10^{-4} M in R 851/26, respectively. Propionyl-CoA seems to be a better substrate for thioesterase, or two specific enzymes are involved in deacylation. The affinity for propionyl-CoA was about 5 times higher than for acetyl-CoA in the low producing strain, and 7 times higher in the strain producing high yield of polifungin. This suggests the occurrence of one enzyme rather than two since the affinity towards both substrates changed similarly on selection of the high producing strains of *Streptomyces noursei* var. *polifungini*.

The regulatory role of thioesterase in biosynthesis of polifungin is evident from the survey of enzymic activities involved in the synthesis of acetyl-CoA and pro-

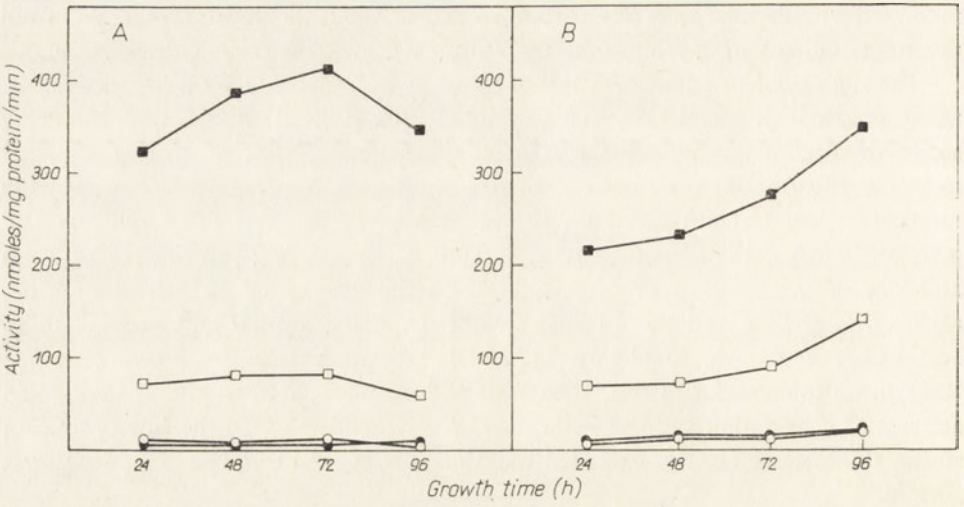

Fig. 2. Time-course of deacylation of acetyl-CoA (○, □) and propionyl-CoA (●, ■) in the high producing R 851/26 (○, ●) and low producing 820/25 (□, ■) strains of *S. noursei* var. *polifungini*, grown on lipid (*A*) and carbohydrate (*B*) media.

pionyl-CoA in *S. noursei* var. *polifungini*, and the negative correlation of thioesterase activity with the size of acyl-CoA pool. In view of the high activity of thioesterase(s) degrading acetyl-CoA and propionyl-CoA, the antibiotic synthesis may be retarded due to the deficiency of the basic building units. This explains the previously proved importance of the "activation system", i.e. non-specific acetate kinase, in the selection of high producers of erythromycin (Raczyńska-Bojanowska *et al.*, 1973). The kinase and phosphotransferase could be regarded as the systems filling up the losses in the pool of acetyl-CoA and propionyl-CoA resulting from thioesterase activity.

Determination of the enzymes hydrolysing acetyl-CoA and propionyl-CoA in the spheroplasts and the cell wall fraction obtained on lysozyme treatment of the 72-h mycelium of 820/25 strain in hypertonic sucrose solution showed (Table 1) that both deacylating activities were localized in the spheroplast fraction.

Table 1

Localization of acetyl-CoA and propionyl-CoA thioesterase activity in mycelium of S. noursei *var.* polifungini

Washed 72-h mycelium of 820/25 strain was treated with lysozyme (1 mg/ml) and EDTA (0.025 M) in 20% sucrose containing 10 mM-2-mercaptoethanol. The solubilized cell wall fraction was separated by centrifugation at 16 000 rev./min. The enzymic activities were assayed as described in Methods and expressed in nmoles/min.

Preparation	Substrate	
	Acetyl-CoA	Propionyl-CoA
Whole lysozyme-treated cells	30 000	127 000
Soluble fraction	780 (3%)	2 760 (3%)
Spheroplasts	21 000 (97%)	75 600 (97%)

On the contrary, acetyl-CoA carboxylase was present in the cell wall fraction (Rafalski & Raczyńska-Bojanowska, 1972). This may indicate compartmentation of the metabolism of acetyl-CoA and propionyl-CoA in the cell of *S. noursei* var. *polifungini*.

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PULA ACYLO-CoA I TIOESTERAZA ACYLO-CoA U *STREPTOMYCES NOURSEI* VAR. *POLIFUNGINI*

Streszczenie

Wielkość puli acylo-CoA u *S. noursei* var. *polifungini* jest związana ze zdolnością do syntezy antybiotyku i jest ujemnie skorelowana z czynnością tioesterazową (hydrolaza acetylo-CoA, EC 3.1.2.1) oraz powinowactwem do acetylo-CoA i propionylo-CoA. Stałe Michaelisa u szczepu niskoprodukcyjnego wynoszą w stosunku do acetylo-CoA 33×10^{-5} M, a do propionylo-CoA 6.6×10^{-5} M, u szczepu wysokoprodukcyjnego odpowiednio 8.5×10^{-4} M i 1.2×10^{-4} M.

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HOMOLANTHIONINE IN FUNGI: ACCUMULATION IN THE METHIONINE-REQUIRING MUTANTS OF *ASPERGILLUS NIDULANS**

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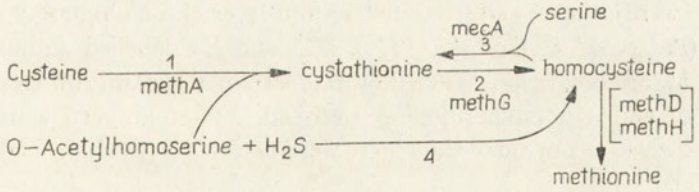
Homolanthionine, a higher homologue of cystathionine, was found to accumulate in the mutants of *Aspergillus nidulans* impaired in the synthesis of methionine from homocysteine. The additional introduction of mutation resulting in a block at cystathionine γ-synthase but not at cystathionine β-synthase abolishes accumulation of both homolanthionine and cystathionine. This suggests a possible participation of cystathionine γ-synthase in homolanthionine synthesis.

L-Homolanthionine ($HOOC \cdot CHNH_2 \cdot (CH_2)_2 \cdot S \cdot (CH_2)_2 \cdot CHNH_2 \cdot COOH$), a higher analogue of cystathionine ($HOOC \cdot CHNH_2 \cdot CH_2 \cdot S \cdot (CH_2)_2 \cdot CHNH_2 \cdot COOH$) was first detected in *E. coli* mutant unable to form methionine from homocysteine (Huang, 1963). The same substance was later found in urine of patients with homocystinuria (Perry *et al.*, 1966), a metabolic disorder caused by a lack of cystathionine β-synthase activity (Mudd *et al.*, 1964; Finkelstein *et al.*, 1964).



Scheme 1. Biosynthesis of cystathionine and homocysteine in *Aspergillus nidulans*. *1*, Cystathionine γ-synthase (EC 4.2.99.9); *2*, β-cystathionase (EC 4.4.1.8); *3*, cystathionine β-synthase (EC 4.2.1.22); *4*, homocysteine synthase. Gene symbols *methH* and *methD* are given in brackets as the biochemical character of these mutants has not yet been determined.

* This work was supported by the Polish Academy of Sciences within the project 09.3.1 and by the U.S. Public Health Service, grant No. 05-001-0.

In both cases accumulation of homolanthionine resulted from an excess of homocysteine: in *E. coli* mutant due to a block in methionine synthesis, in homocystinuria owing to a blocked methionine catabolism.

We have found that homolanthionine accumulates in the two methionine-requiring mutants of *Aspergillus nidulans*, *methH2* and *methD10* (Scheme 1). Both mutants are unable to synthesize methionine from homocysteine (Gajewski & Litwińska, 1968): the first one probably due to the impairment of transmethylating enzyme, the second because of a lack of the methyl donor (Paszewski & Grabski, 1975).

MATERIALS AND METHODS

Biological material. The following strains of *Aspergillus nidulans* were used in this work: *methH2 pyroA4y*, *methD10 pyroA4y*, *methG55 pyroA4y*, *methA17 pyroA4y*, *mecA1 biA1 anA1* and *pyroA4y*. As illustrated in Scheme 1, the mutants *methA17* and *methG55* lack cystathionine γ-synthase and β-cystathionase, respectively; *methH2* and *methD10* are blocked between homocysteine and methionine (Paszewski & Grabski, 1975); *mecA1* is devoid of cystathionine β-synthase (Pieniążek *et al.*, 1973*a*, *b*).

The following recombinant strains were isolated: *methD10 methA17*, *methD10 methG55* and *methD10 mecA1*. The strain *pyroA4y*, referred to as the wild type, was used as the reference strain.

meth, *pyro*, *bi* and *an* denote the requirements for methionine, pyridoxine, biotin and aneurine, respectively; *mec* denotes methionine catabolism.

Media and culture conditions. Liquid minimal medium (MM) containing mineral salts as described by Cove (1966), microelements as in the Difco Yeast Base without amino acids and with glucose as a carbon source was modified or supplemented as indicated. Cultures were started by inoculation of 150 ml of the medium in 250 ml Erlenmeyer flasks with a heavy conidial suspension, and incubated at 30 - 31°C in a rotary shaker for indicated periods of time.

Isolation and separation of ^{35}S- *and* ^{14}C-*labelled amino acids.* Mycelia grown in the presence of $Na_2{}^{35}SO_4$ (1.5 - 2 μCi/mmole) or [^{14}C]homoserine (0.33 μCi/mmole) were harvested on the filter paper, washed thoroughly with water and blotted. Amino acids were isolated and separated by thin-layer chromatography as described previously (Paszewski & Grabski, 1974). ^{35}S- and ^{14}C-labelled amino acids were located by autoradiography. When non-radioactive standards of L-cystathionine, L-methionine and L-homolanthionine were cochromatographed with radioactive material, the positions of these thioethers were detected with chloroplatinic reagent (Wong, 1971).

Identification of homolanthionine. The amino acid fraction corresponding to 300 mg of mycelial dry weight, isolated from *methD10* grown in MM medium supplemented with 1 mM-betaine, was separated on Dowex 50(H^+), then applied on five chromatographic plates (20 × 20 cm) covered with MN-300 cellulose powder. The plates were developed in butan-2-ol - acetic acid - water (12:3:5, by vol.) system. Positions of cystathionine and presumed homolanthionine were determined by

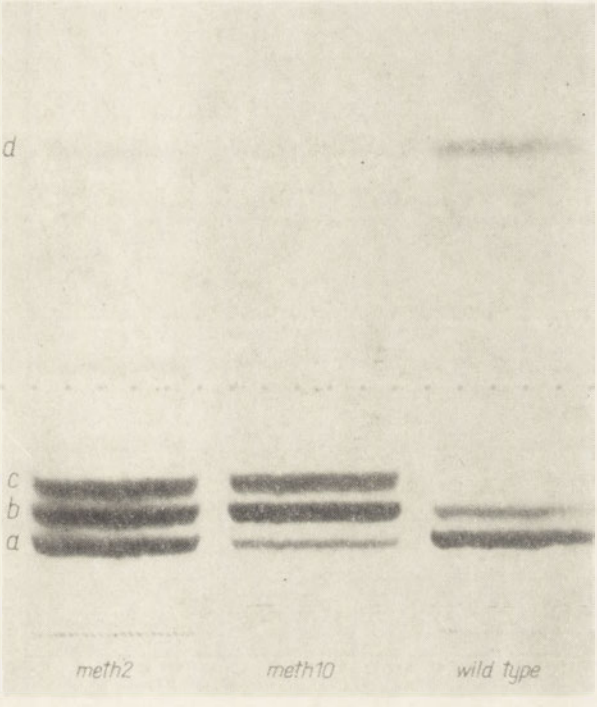


Fig. 1. Autoradiography of ^{35}S-labelled compounds from *methH2*, *methD10* and the parental strain. Mycelia grown in MM supplemented with L-methionine (2 mM) at 30°C for 15 h were transferred to MM containing 0.4 mM-$Na_2{}^{35}SO_4$ (5×10^8 c.p.m./mmole) and grown for an additional 8 h. The amino acids retained on Dowex 50 (H^+) were then analysed by thin-layer chromatography (Paszewski & Grabski, 1974). For each strain, material corresponding to 20 mg of dry mycelium was analysed. *a*, Glutathione (oxidized); *b*, cystathionine; *c*, homolanthionine; *d*, methionine.

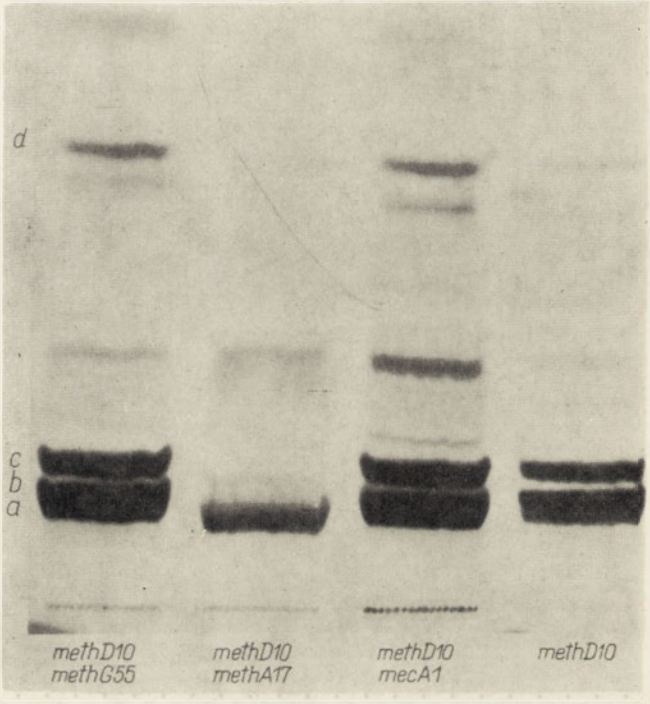


Fig. 2. Autoradiography of ^{35}S-labelled compounds in water extracts from mycelia of *methD10 methG55*, *methD10 methA17*, *methD10 mecA1* and *methD10* grown in the presence of radioactive sulphate. Experimental conditions as given in the legend to Fig. 1. The amount of radioactive material applied on the plate corresponded to 20 mg of dry mycelium.

spraying the edge of each chromatogram with chloroplatinic reagent. The bands of cellulose powder corresponding to these amino acids were scraped off and suspended in 5 ml of water. About 200 mg of Raney nickel in 0.3 ml of water was then added and the mixture was gently boiled under reflux for 3 h. The mixture was filtered and amino acids were separated from the filtrate on Dowex 50 (H^+) column (0.5 × 7 cm) and eluted with 2 M-NH_4OH. The eluate was evaporated and the residue dissolved in 100 µl of H_2O; 50 µl aliquots corresponding to cystathionine and presumed homolanthionine were applied on a cellulose chromatographic plate together with alanine and α-aminobutyric acid as standards. The chromatograms were developed in the above-mentioned system using ninhydrin for detection of amino acid spots.

Reagents. L-Homolanthionine was a generous gift from Dr. S. H. Mudd. L-Cystathionine, L-alanine and L-α-aminobutyric acid were obtained from Calbiochem (Los Angeles, Calif., U.S.A.), L-methionine and betaine from Merck (Darmstadt, G.F.R.). L-[U-^{14}C]Homoserine (32 mCi/mmole) was purchased from The Radiochemical Centre (Amersham, England). $Na_2{}^{35}SO_4$ was obtained from the Institute of Nuclear Research (Świerk, Poland).

All inorganic compounds were reagent grade products.

RESULTS

Two *Aspergillus nidulans* mutants, *methH2* and *methD10*, impaired in the synthesis of methionine from homocysteine accumulate large amounts of cystathionine and a compound identified as homolanthionine (Fig. 1). Both mutants are leaky since some radioactivity was found in methionine as shown on the chromatograms. The occurrence of cystathionine in *Aspergillus nidulans* has been reported by Paszewski & Grabski (1974). Identity of homolanthionine was proved by its mobility identical with that of authentic L-homolanthionine in different solvent systems (*n*-butanol - acetic acid - water, 12:3:5, by vol; isopropanol - formic acid - water, 75:12.5:12.5, by vol.), and by analysis of the reaction product with Raney nickel. Removal of sulphur with Raney nickel yielded only α-aminobutyric acid as shown by chromatography. Under the same conditions, cystathionine yielded both alanine and aminobutyric acid.

The origin of homolanthionine could be established by means of radioactivity distribution in homolanthionine and cystathionine. If homolanthionine is formed by condensation of homocysteine with homoserine (or *O*-acetylhomoserine), the homolanthionine : cystathionine radioactivity ratio should be twice as high when mycelium is grown in the presence of radioactive homoserine than when ^{35}S is administered as sulphate (homolanthionine contains two 4-carbon chains and cystathionine only one; both compounds, on the other hand, contain one atom of sulphur). The results presented in Table 1 are in agreement with this supposition: the homolanthionine : cystathionine radioactivity ratio is 1.91 times higher in the mycelium grown in the presence of radioactive homoserine than in the presence of $^{35}SO_4$ — this value being close to the theoretical one.

Table 1

Distribution of ^{35}S and ^{14}C in cystathionine and homolanthionine

MethD10 was grown in MM medium containing 0.1 mM-L-methionine at 30°C for 16 h. Then identical portions of the mycelium (about 200 mg wet weight) were suspended in 30 ml of MM containing 0.2 mM-L-threonine and either 0.2 mM-$Na_2{}^{35}SO_4$ (1.9 μCi/mmole) and 0.2 mM-L-homoserine, or 0.2 mM-L-[U-^{14}C]homoserine (0.33 μCi/mmole) and 0.2 mM-Na_2SO_4. Both cultures were incubated at 30°C for 5 h. Radioactive amino acids were isolated and separated as described by Paszewski & Grabski (1974).

Amino acid*	^{35}S (c.p.m.)	^{14}C (c.p.m.)
Homolanthionine	3147	789
Cystathionine	8498	1108
Ratio $\frac{\text{homolanthionine}}{\text{cystathionine}}$	0.37	0.71

* Material corresponding to 20 mg of dry mycelium was analysed.

It was of considerable interest to know whether homolanthionine is synthesized by a specific enzyme or by an enzyme involved in the metabolism of sulphur amino acids, such as cystathionine γ- and β-synthases. Therefore we have isolated double mutants carrying *methD10* and alternatively mutations *methA17*, *mecA1* or *methG55*, with a block in cystathionine γ-synthase, cystathionine β-synthase or β-cystathionase, respectively (see Scheme 1). The last mutation was introduced to see whether homocysteine is formed efficiently enough by the alternative pathway directly from *O*-acetylhomoserine and sulphide, when the main pathway is impaired. It is clear from Fig. 2 and Table 2 that homolanthionine accumulates in *methD10* carrying an additional block at β-cystathionase or cystathionine β-synthase but not at cystathionine γ-synthase.

DISCUSSION

This is, to our knowledge, the third report on the occurrence of homolanthionine in an organism and the first in fungi. The identification procedure used did not exclude the possibility that the studied compound might be β-methylcystathionine, the thioether which would result from condensation of homocysteine and threonine. Such a possibility was discussed by Perry *et al.* (1966) in their paper on homolanthionine in patients with homocystinuria. However, if threonine were a precursor of the studied compound, the homolanthionine : cystathionine radioactivity ratio in [^{14}C]homoserine-grown mycelium *versus* $^{35}SO_4$-grown mycelium should be less than 2 since cold threonine present in the growth medium (see Table 1) would dilute considerably radioactive threonine formed from [^{14}C]homoserine.

Experiments with double mutants excluded the role of cystathionine β-synthase in the formation of homolanthionine. They indicate also that under our experimental conditions enough homocysteine is synthesized by the alternative pathway involving homocysteine synthase (Scheme 1) to ensure large accumulation of homo-

Table 2

Accumulation of ^{35}S-*labelled amino acids in the wild type and the* Aspergillus nidulans *mutants grown in the presence of* $^{35}SO_4$

Experimental conditions as described in the legend to Fig. 1. The results are expressed in μmoles/g dry wt.

Amino acid	wild type	*methH2*	*methD10*	*methD10 mecA1*	*methD10 methA17*	*methD10 methG55*
Glutathione	1.66	1.34	0.58	1.30	0.82	1.66
Cystathionine	0.87	1.71	1.81	2.03	0.39	3.17
Homolanthionine	traces	1.26	1.25	2.55	traces	2.24
Methionine	0.52	0.24	0.15	0.44	0.15	0.49
Total ^{35}S	6.60	9.93	7.61	13.4	1.7	13.3

lanthionine in spite of a block at β-cystathionase. These conditions favour derepression of homocysteine synthase since the transfer of mycelia from methionine-containing medium to minimal medium results in a relative shortage of cysteine which represses homocysteine synthase until *de novo* synthesis of cysteine from sulphate is reestablished. On the other hand, introduction to *methD10* of a mutation causing a block in cystathionine γ-synthase prevented accumulation of both cystathionine and homolanthionine. It is possible, however, that much less homocysteine is formed by the alternative pathway when there is a block at cystathionine γ-synthase than at β-cystathionase, as in the first case there is a surplus of cysteine which was found to repress homocysteine synthase (Paszewski & Grabski, 1974). The fact, however, that the double mutant *methD10 methA17* forms almost the same amount of radioactive methionine as *methD10* (Table 2) suggests that it is rather an impairment of cystathionine γ-synthase than a shortage of homocysteine which accounts for the lack of homolanthionine accumulation in the double mutant. Thus, it might be assumed that homolanthionine is synthesized by cystathionine γ-synthase in the following reaction:

O-Acetylhomoserine + homocysteine → homolanthionine + acetic acid,

where homocysteine substitutes for the normal substrate, that is cysteine.

We wish to thank Dr. K. Raczyńska-Bojanowska for critical reading of the manuscript.

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HOMOLANTIONINA W GRZYBACH: ZWIĘKSZENIE PULI U MUTANTÓW *ASPERGILLUS NIDULANS* WYMAGAJĄCYCH METIONINY

Streszczenie

Stwierdzono nagromadzanie się homolantioniny, wyższego homologu cystationiny, w mutantach *Aspergillus nidulans* niezdolnych do syntezy metioniny z homocysteiny. Po wprowadzeniu dodatkowej mutacji powodującej blok w γ-syntazie cystationinowej, lecz nie w β-syntazie cystationinowej, nie występuje nagromadzanie ani homolantioniny ani cystationiny. Sugeruje to udział γ-syntazy cystationinowej w syntezie homolantioniny.

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RECENZJE KSIĄŻEK

CHEMICAL AND BIOCHEMICAL APPLICATIONS OF LASERS (C. Bradley Moore, ed.) Vol. I, xi+398 pages, price $ 29.50, Published November 1974, Academic Press, New York.

This is probably the first volume which attempts to review in a comprehensive manner the variety of applications of laser sources in the fields of chemistry, biochemistry and related fields of research. Its appearance is very timely since many laser systems have now been developed to the point of being valuable tools in research and, in many instances, are commercially available or incorporated into other commercially available instruments.

The present volume consists of 11 chapters, each of which is in essence a review of a given field of application by specialists working in these fields. They cover such topics as Raman spectroscopy, energy flow in polyatomic molecules, stimulation of chemical reactions with laser radiation, kinetic studies of very rapid chemical reactions in solution, rapid reactions in photobiology, etc. Of special interest to all readers, whether graduate students or full-time research workers, is the 1st chapter, consisting of a general introduction to the physics of lasers and to the properties of various practical laser systems. The detailed subject index for a collective volume of this nature is to be commended.

It is to be regretted that the volume does not contain a chapter on applications to light scattering. The advent of lasers has enormously stimulated renewed interest in Rayleigh scattering and its use in studies on the optical anisotropy and electronic structure of molecules of biological interest. Since this is Volume I in a projected series, it is to be hoped that Volume II will include a chapter on this subject.

This volume, and succeeding ones in this series, should prove useful to many research workers in the fields of chemistry, physical chemistry, polymer chemistry, spectroscopy, photochemistry and photobiology, and various fields of biochemistry and biophysics.

David Shugar

METHODS IN ENZYMOLOGY, Vol. XXXVIII (HORMONE ACTION). Part C, entitled CYCLIC NUCLEOTIDES (J. G. Hardman & B. W. O'Malley, eds.), xxi+454 pp. Academic Press, New York, 1974, price $ 29.50.

This addition to the series of volumes originated by S. P. Colowick and N. O. Kaplan may at first sight appear somewhat unusual. In fact the editors in their Preface suggest that some may consider it "arbitrary categorization" to devote an entire volume to nucleoside 3′,5′-cyclic monophosphates, entirely apart from preceding volumes which have covered nucleotide methodologies.

It requires no more than a superficial examination of the Table of Contents, and of the list of contributors, to realize that this volume represents an important practical contribution to all those engaged in research in the fields of endocrinology, hormone action and control, enzymology, and other related fields. The timeliness and utility of this text are further testified to by the extraordinary rapid growth of the literature in this field during the past 5 to 6 years.

The book is divided into six Sections all dealing with practical techniques on (I) Extraction and Purification of Cyclic Nucleotides, (II) Assay of Cyclic Nucleotides, (III) Biosynthesis of Cyclic

Nucleotides, (IV) Degradation of Cyclic Nucleotides, (V) Cyclic Nucleotide-Dependent Protein Kinases and Binding Proteins, (VI) Synthetic Derivatives of Cyclic Nucleotides and their Precursors.

It is symptomatic of the current rapid rate of development of research that the contributions to this volume treat in some detail the individual enzyme systems associated with cAMP and cGMP; in fact the latter, which is relatively new, is given broader treatment than cAMP in Section II on methods of assay. This is perhaps due to the widespread use of Gilman's procedure for cAMP assay, as compared to the relatively new methods developed for assaying cGMP, present in tissues at concentration levels one to two orders of magnitude lower than cAMP. Although an accurate account is given of the preparation and properties of cGMP-dependent kinase, the date of publication apparently precluded inclusion of the new method of Kuo (*Proc. Nat. Acad. Sci., U.S.*, **71**, 4037 - 4041, 1974) for isolation of this enzyme from mammalian tissues.

Section VI on Synthetic Derivatives of Cyclic Nucleotides illustrates once again the present-day difficulties which even collective volumes have in keeping pace with the rate of modern research. Although this section includes a description of the synthesis of the fluorescent cAMP analogue, 1,N^6-ethenoadenosine-3′,5′-monophosphate, it does not adequately reflect the extensive research activity in this field in 1974.

It is most appropriate that the frontispiece contains a photograph of, and a dedication written by Joel G. Hardman to, Earl W. Sutherland, Jr., who virtually founded the field of cyclic nucleotide research and its role in fundamental regulatory mechanisms, and whose untimely death preceded the publication of this volume by several months.

Like other volumes in this series, this one contains good author and subject indexes, which are of considerable value to the prospective reader. It should be not only a useful adjunct to many libraries, but also a handy text to have beside the lab bench.

David Shugar

E. A. Newsholme and C. Start, REGULATION IN METABOLISM. John Wiley and Sons, London, New York, Sydney, Toronto 1973; str. vii+349.

Książka jest, jak piszą autorzy we wstępie, przeznaczona dla studentów i istotnie można ją uważać za znakomity podręcznik omawiający zagadnienia regulacji metabolizmu u zwierząt wyższych. W dwóch pierwszych rozdziałach omówiono własności enzymów regulatorowych oraz ogólne mechanizmy regulacji, przy czym przedyskutowano nie tylko aspekty teoretyczne, lecz również eksperymentalne metody umożliwiające „identyfikację" enzymów regulatorowych oraz określenie ich roli w regulacji badanych szlaków przemian. Każdy następny rozdział rozpoczyna się wstępem wprowadzającym w zagadnienie regulacji przemiany energetycznej w tkance będącej przedmiotem dyskusji w danym rozdziale. W kolejnych rozdziałach omówiono regulację metabolizmu węglowodanów i tłuszczów w mięśniach, tkance tłuszczowej i wątrobie. Materiał został uporządkowany według założeń omówionych we wstępnych rozdziałach. Dzięki temu układowi, licznym krótkim streszczeniom rekapitulującym omawiane zagadnienia, a także wielu schematom — podany materiał jest zrozumiały i bardzo łatwy do zapamiętania. Na uwagę zasługują również te fragmenty książki, które omawiają znaczenie fizjologiczne enzymów regulatorowych. Książka zasługuje na szerokie rozpowszechnienie w środowisku akademickim i lekarskim i warto byłoby ją przetłumaczyć na język polski.

Konstancja Raczyńska-Bojanowska

METHODICUM CHIMICUM. Band 6: C-N-VERBINDUNGEN (redaktor tomu F. Zymalkowski, redaktor całości F. Korte). Georg Thieme Verlag, Stuttgart—Academic Press, New York, San Francisco, London 1974; str. 864, cena DM 390, —.

Oceniana książka stanowi szósty tom jedenastotomowego dzieła zatytułowanego „Methodicum Chimicum". Dotyczy ona określonych syntez połączeń węgla i azotu najważniejszych grup związków organicznych.

W pierwszych trzech rozdziałach przedstawiono syntezy aromatycznych i alifatycznych związków nitrowych i nitrozowych oraz hydroksylamin. Osobne omówienie poświęcono pochodnym hydrazyny. Kolejno rozpatrzono zagadnienia związane z syntezą i przekształceniami alifatycznych i aromatycznych azo- i azoksypochodnych oraz soli aromatycznych związków dwuazoniowych i alifatycznych związków dwuazowych. Pobieżnie uwzględniono otrzymywanie azydków organicznych i oksymów. Kilka rozdziałów książki poświęcono syntezie takich klas związków organicznych jak nitryle, izonitryle, cyjaniany i tiocyjaniany. Obszerną część tego tomu stanowią opracowania dotyczące preparatyki amin, alkylenoimin, aminokwasów i czwartorzędowych zasad amoniowych.

Każdy rozdział książki pozwala zapoznać się z krytycznym przeglądem oryginalnych i stosowanych metod syntezy omawianych związków organicznych. Opisano także teoretyczne podstawy głównych operacji stosowanych w preparatyce organicznej syntetyzowanego związku. Ułatwia to dokonanie wyboru celowego postępowania zdążającego do syntezy zamierzonego związku bez sięgania do źródłowych pozycji literaturowych. Wartość książki leży również w tym, że uwzględnia ona zasadnicze reakcje uboczne towarzyszące syntezie określonego związku. Ujęcie niektórych syntez związków organicznych tak, aby możliwym stało się zaniechanie izolacji szczególnie niebezpiecznych produktów, pozwala dokonać wyboru postępowania uwzględniającego również bezpieczeństwo pracy. Zestaw metod zaczerpnięto z podstawowych archiwalnych prac cytowanych na każdej stronie i opracowań monograficznych z zakresu preparatyki organicznej umieszczonych na końcu poszczególnych rozdziałów.

Ogromnie bogata literatura źródłowa, sięgająca dziesięciu tysięcy pozycji, w którą zaopatrzono ten tom, pozwala znaleźć łatwo oryginalne prace o istotnym znaczeniu dla poszczególnego etapu planowanej syntezy organicznej.

Oceniany tom szósty „Methodicum Chimicum" stanowi cenną pozycję nie tylko dla specjalistycznej grupy chemików organików pracujących w laboratoriach naukowych, lecz także dla szerokiego grona chemików zajmujących się przemysłową syntezą związków organicznych.

Leon Żelewski

BIOPHYSICS, vol. 3: MOLECULAR BIOENERGY, part 1. A. A. Yasaytis. Redaktor wydania: V. P. Skulaczev. G. K. Hall and Co. Boston, Massachusetts, 1974, str. 130.

„Bioenergetyka molekularna" jest kolejnym tomem seryjnego wydawnictwa publikowanego przez Wszechzwiązkowy Instytut Informacji Naukowej i Technicznej ZSSR. Część 1, której podtytuł brzmi „Przetwarzanie energii w mitochondriach", jest obszernym przeglądem dorobku ostatnich lat 10 w dziedzinie procesów oksydoredukcji w mitochondriach i ściśle z nimi sprzężonych reakcji prowadzących do syntezy ATP. Poszczególne rozdziały zawierają ponadto najnowsze wiadomości dotyczące budowy błon mitochondrialnych i ich funkcji w transporcie metabolitów. Bardzo obszernie i szczegółowo potraktowano rozdział o tzw. chemiosmotycznej teorii sprzężenia oksydacyjnej fosforylacji. Wynika to głównie z własnego, eksperymentalnego zaangażowania autora w tej dziedzinie.

Bardzo bogaty (około 700 pozycji) zestaw cytowanej literatury udostępnia wiele pozycji rosyjskich, na ogół mniej znanych czytelnikom z krajów anglosaskich. Przekład angielski (autor przekładu nie jest wymieniony) całego artykułu zawiera jednak dużo usterek, począwszy od niezbyt ścisłego tytułu, bowiem przyjęty termin angielski brzmi: Molecular Bioenergetics a nie Bioenergy.

Na grzbiecie książki podano „Skulayeva" zamiast „Skulachev". W tekście spotyka się także biochemiczne terminy przetłumaczone nieprawidłowo, a pisownia nazw chemicznych niektórych związków jest błędna. Z tych względów czytelnikowi polskiemu należy raczej polecić wydanie oryginalne w języku rosyjskim.

Anna Wojtczak

W. Friedrich, VITAMIN B_{12} UND VERWANDTE CORRINOIDE. Georg Thieme Verlag, Stuttgart 1975; str. XI+360, cena DM 168,-.

Omawiana książka została opublikowana jako III tom serii „Fermente, Hormone, Vitamine", wydawanej przez R. Ammona i W. Dirscherla.

Fakt, że jednej witaminie poświęcono w tym wydawnictwie odrębny tom, świadczy o pewnej niezwykłości problematyki witaminy B_{12}. Złożyło się na to szereg przyczyn. Witamina B_{12} należy do substancji działających w najmniejszych ilościach w porównaniu z innymi witaminami. Występuje też w przyrodzie ożywionej w bardzo niskich stężeniach; jest syntetyzowana tylko przez niektóre drobnoustroje; nie wymagają jej rośliny wyższe; jest pierwszym odkrytym naturalnym związkiem organometalicznym zawierającym kobalt. W przyrodzie występują bardzo liczne jej pochodne, z których szereg także przejawia funkcje witaminowe, względnie wzrostowe dla pewnych mikroorganizmów. Mechanizm działania wit. B_{12} w dużej mierze zależy od metaloorganicznego wiązania C-Co występującego w formie koenzymatycznej witaminy. Wit. B_{12} wymaga dla swego transportu w organizmach zwierzęcych specyficznych białek itd, itd. Nic więc dziwnego, że od czasu izolacji wit. B_{12} w 1948 r. ukazało się przeszło 12 000 publikacji na temat korynoidów.

Udostępnienie tego bogatego materiału wymagało jego uporządkowania i selekcji. Trudu tego podjął się autor książki, mający sam wieloletnie doświadczenie w badaniach związków korynowych. Książka jest pierwszą tak obszerną monografią korynoidów i zawiera całokształt wiadomości o tych związkach.

Materiał książki jest zebrany w trzech rozdziałach. Pierwszy z nich jest poświęcony metodom izolacji, opisowi właściwości fizyko-chemicznych, chemicznej syntezie oraz analizie korynoidów; drugi biosyntezie i występowaniu tych związków; wreszcie trzeci (obejmujący połowę objętości książki) zawiera materiał dotyczący roli wit. B_{12} w odżywianiu, przemianach biochemicznych oraz w medycynie.

Nadto autor przedstawia na wstępie krótkie wprowadzenie do nomenklatury korynoidów zalecanej przez IUPAC-IUB.

W rozdziale pierwszym znajdzie czytelnik, obok krótkiego opisu metod stosowanych do izolacji i oczyszczania korynoidów z materiału biologicznego, wyczerpujący opis bardzo licznych pochodnych korynowych występujących w przyrodzie. Przedstawiając ich właściwości fizyko-chemiczne autor książki zestawia między innymi widma absorpcyjne aż 149 pochodnych. Bardzo dużo miejsca zajmuje w tym rozdziale opis właściwości chemicznych korynoidów, zarówno samego pierścienia korynowego, jego łańcuchów bocznych, jak i własności wynikających z obecności w nich atomu kobaltu. Rozdział zawiera także zwięzłą informację o pełnej syntezie chemicznej wit. B_{12} dokonanej ostatnio przez grupę prof. Eschenmosera w Zürichu oraz prof. Woodwarda w Cambridge, Massachusetts.

Dział analityczny (bez analizy biochemiczno-klinicznej) jest reprezentowany raczej skromnie.

W rozdziale drugim autor omawia obecny stan wiedzy o biosyntezie korynoidów. Jest to zagadnienie jeszcze otwarte, szczególnie od strony enzymologicznej, ale i część chemiczna zagadnienia, występowanie poszczególnych reakcji, ich kolejność, nie jest jeszcze wyjaśniona. Przedstawione w tym rozdziale dane tyczące występowania korynoidów w przyrodzie są bardzo zwięzłą, tabelaryczną prezentacją danych literaturowych.

Rozdział trzeci zaczyna się od omówienia zapotrzebowania wit. B_{12} przez organizmy zwierzęce oraz omówienia biologicznych metod oznaczania witaminy. Następnie autor przedstawia znane biochemiczne mechanizmy działania wit. B_{12}. Witamina B_{12}, jak wiadomo, bierze udział w reakcjach enzymatycznych w formie pochodnej Co-5′-dezoksyadenozylowej (dla większości poznanych reakcji) lub pochodnej Co-5′-metylowej (w reakcji syntezy metioniny i ewentualnie kilku innych).

Około 20 stron swej książki poświęca autor zagadnieniu transportu wit. B_{12} w organizmach wyższych, a więc tzw. czynnikowi wewnętrznemu, niezbędnemu do resorpcji wit. B_{12} z przewodu pokarmowego, oraz trzem białkowym transkobalaminom uczestniczącym w transporcie witaminy np. w organizmie ludzkim. W dalszym ciągu tego rozdziału prezentuje autor dane dotyczące zagadnienia awitaminozy B_{12} oraz przyczyn występowania tej awitaminozy. Część tę uzu-

pełnia opisem tych reakcji enzymatycznych, które są uzależnione od niedoboru wit. B_{12} a także kwasu foliowego w organizmie ludzkim. Wreszcie omawia obszerny materiał, jaki zebrano dotychczas w badaniach biologicznych różnych pochodnych korynowych, w tym także otrzymywanych w drodze modyfikacji chemicznych. Rozdział ten zamyka autor opisem stosowanych dla celów klinicznych metod oznaczania wit. B_{12} i białek wiążących witaminę B_{12}.

Książka zawiera bogatą literaturę przedmiotu, obejmującą około 2000 pozycji i czasokres do połowy 1974 roku. W cytowanej literaturze znajduje się większość prac wykonanych w pracowniach polskich, nawet tych, które, pochodząc z wcześniejszego okresu, były publikowane w języku polskim. Dobra znajomość języka polskiego ze strony autora książki pozwoliła ustrzec te prace przed zapomnieniem.

Omawiana książka winna się znaleźć w rękach tych wszystkich, którzy interesują się problematyką witamin, a w szczególności związkami korynowymi.

Jerzy Pawełkiewicz

T. Dévényi and J. Gergely, AMINO ACIDS, PEPTIDES AND PROTEINS. Akadémiai Kiadó, 1974; stron 343.

Autorzy omawianej książki zebrali i przedstawili techniki stosowane w badaniach nad białkami. Podali w sposób zwięzły i przystępny ogólny opis metod, procedurę postępowania oraz przegląd niezbędnej aparatury. Poszczególne opisy technik obok krótkiego wprowadzenia teoretycznego zawierają wiele cennych uwag praktycznych. Szczególnym walorem są własne obserwacje i doświadczenia autorów. Każdy rozdział zawiera bogate piśmiennictwo pozwalające na rozszerzenie teoretycznych i praktycznych wiadomości.

Pierwsze rozdziały książki poświęcone są technikom stosowanym w badaniach nad białkami natywnymi. Autorzy podają różne sposoby rozdziału białek. Omawiają elektroforezę na bibule, skrobi, agarze, octanie celulozy oraz żelu poliakrylamidowym. Wiele uwagi poświęcają badaniom immunologicznym, szeroko opisując immunoelektroforezę i jej zastosowanie. Chromatografia jonowymienna i sączenie na Sephadexach jest tematem następnych rozdziałów.

Dalsza część książki jest poświęcona wstępnym badaniom nad strukturą cząsteczki białka. Podano chemiczne i enzymatyczne metody hydrolizy białek, rozdział i warunki izolowania uzyskanych peptydów. Oznaczanie składu aminokwasów, analiza grup końcowych oraz badanie sekwencji aminokwasów jest przedmiotem ostatnich rozdziałów.

Omawiana książka dostarcza cennych wiadomości praktycznych na temat badania białek, wydaje się jednak, że część podanych przez autorów technik należy już do metod klasycznych, dostępnych w podręcznikach akademickich. Szkoda, że autorzy nie przedstawili technik nowocześniejszych, jak metoda ogniskowania w gradiencie pH lub chromatografia powinowactwa. Metody te rozwinęły się w ciągu ostatnich lat i są stosowane z dużym powodzeniem w badaniach nad izolowaniem i charakterystyką białek.

Mimo wspomnianych zastrzeżeń, omawiana książka stanowi wartościową pozycję i będzie niewątpliwie przydatna dla tych, którzy rozpoczną badania nad budową białek.

Zofia Porembska

HORIZONS IN BIOCHEMISTRY AND BIOPHYSICS, vol. 1. E. Quagliariello, F. Palmieri and T. P. Singer, eds. Addison-Wesley Publishing Company, Inc., Advanced Book Program. Reading, Massachusetts - London - Amsterdam - Don Mills, Ontario - Sydney - Tokyo, 1974; str. xii+344, cena $ 13.50.

Omawiana książka pt. *Horizons in Biochemistry and Biophysics* stanowi nowy typ seryjnego wydawnictwa, mającego stworzyć pomost łączący różne dziedziny nauk biologicznych. Celem, jaki przyświeca redaktorom tego wydawnictwa, jest zwrócenie uwagi młodych pracowników naukowych, wykładowców i osób pracujących zawodowo w różnych dziedzinach nauk biologicznych i fizyki —

łącznie z medycyną — na najważniejsze twórcze koncepcje, osiągnięcia metodyczne i przełomowe odkrycia jakich dokonano w biochemii i biofizyce w ostatnim dziesięcioleciu. Daje się bowiem odczuć pilna potrzeba re-waluacji ogólnie przyjętych teorii, ukierunkowania badań na przyszłość i stworzenia możliwości szybkiego korzystania z nowych odkryć w pokrewnych dziedzinach wiedzy.

Na to, aby nowa seria spełniła pokładane w niej nadzieje, artykuły — zdaniem redaktorów — muszą być dobrze napisane, nie obciążone specjalistycznym żargonem, nadmierną dokumentacją i bibliografią, tak aby ich treść była równie dostępna dla studentów wyższych lat studiów uniwersyteckich (np. z genetyki), lekarzy i zawodowych chemików. Redaktorzy zapewniają, iż dołożą wszelkich starań, aby znaleźć na obu kontynentach — w Europie i Ameryce — ludzi o dużym autorytecie naukowym, których główną pasją byłoby szerzenie wiedzy a nie przedstawianie własnych osiągnięć naukowych pod kątem ich priorytetu.

Omawiany pierwszy tom zawiera dziewięć doskonałych artykułów naukowych pióra czołowych biochemików i biofizyków. O zagadnieniach poruszanych w tym tomie z serii *Horizons in Biochemistry and Biophysics* dadzą najlepsze wyobrażenie niżej zamieszczone tytuły artykułów: 1. Superoxide and Evolution (Irwin Fridovich), 2. Molecular Nature of Isozymes (William C.Kenney), 3. Covalently Bound Flavin Coenzymes (Edna B. Kearney & William C. Kenney); 4. Biochemical Foundations of Preventive Medicine: The Study of Abnormal Enzymes (David Zakim); 5. Membrane Fluidity and the Regulation of Membrane-Bound Enzymes (Donald A. Vessey & David Zakim); 6. Metabolic Regulatory Functions of Oxalacetate (Brian A. C. Ackrell); 7. Multiple Patways of NADH Oxidation in the Mitochondrion (J. M. Palmer & J. O. D. Coleman); 8. The Quest for Coupling Site I (Thomas P. Singer & M. Gutman); 9. Ferredoxins and Photosynthesis (Daniel I. Arnon & Bob B. Buchanan).

Książka trzyma w napięciu uwagę czytającego, gdyż zarówno tok jak i treść wykładu działa na wyobraźnię i pobudza do myślenia. Artykuły są pisane prostym, pięknym językiem naukowym, bez zbytnich uproszczeń, fakty są przedstawiane bez balastu szczegółów, tak że na plan pierwszy wysuwa się idea, wyłaniają się metody i zarysowuje się obraz całości problemu — jego światła i cienie.

Należy życzyć sobie, aby ten i następne tomy z nowej serii *Horizons in Biochemistry and Biophysics* pod redakcją E. Quagliariello, F. Palmieri and T. P. Singer znalazły się w bibliotekach, jak również były dostępne dla indywidualnych prenumeratorów i dotarły do rąk pracowników naukowych i studentów wyższych lat studiów uniwersyteckich różnych dziedzin nauk biologicznych i lekarskich.

Redaktorom nowej serii wydawniczej i autorom poszczególnych artykułów należą się słowa uznania za podjętą inicjatywę i położony trud, aby wypełnić lukę pomiędzy piśmiennictwem ściśle fachowym i specjalistycznym a wydawnictwami popularnymi.

Wanda Mejbaum-Katzenellenbogen

M. Friedman, THE CHEMISTRY AND BIOCHEMISTRY OF THE SULFHYDRYL GROUPS IN AMINO ACIDS, PEPTIDES AND PROTEINS. Pergamon Press, Oxford, New York, Toronto, Sydney, Braunschweig 1973; str. vii+485; cena 7.5 £

Grupy -SH biorą udział w tak wielu różnorodnych procesach biologicznych, że zebranie przez Friedmana danych dotyczących własności chemicznych i biochemicznych tych grup jest bardzo cenne nie tylko dla biochemików, lecz także dla farmakologów, lekarzy i ludzi związanych z przemysłem spożywczym i tekstylnym. Obszerne omówienie własności chemicznych w 11 rozdziałach monografii pozwala na zrozumienie reaktywności grup sulfhydrylowych, wynikającej najprawdopodobniej z polarnego układu elektronów siarki. Jakkolwiek tylko 4 rozdziały autor poświęca zagadnieniom syntezy i metabolizmu grup sulfhydrylowych, to jednak dzięki dyskusji własności redukujących, jonizacji oraz zdolności chelatowania metali w odniesieniu do układów biologicznych czytelnik jest bardzo dobrze wprowadzony w mechanizm oddziaływania grup sylfhydrylowych w organizmach żywych. W taki sam sposób ujęto omówienie reakcji addycji, rozszczepienia i alkilacji, stanowiących podstawę reakcji związków tiolowych, katalizowanych przez enzymy. Mono-

grafię zamyka rozdział poruszający aspekty farmakologiczne i medyczne związane ze zmianami, jakim podlegają grupy sulfhydrylowe w chorobach tkanki łącznej, dystrofii mięśni, zaburzeniach mózgowych oraz w wyniku działania stosowanych w terapii środków diuretycznych, hormonalnych, geriatrycznych, antybiotyków i innych leków. Korzystanie z bogato cytowanej literatury ułatwia bardzo starannie przygotowany indeks rzeczowy i indeks autorski.

Konstancja Raczyńska-Bojanowska

MTP Interational Review of Science: CHEMISTRY OF MACROMOLECULES. Biochemistry, series one, vol. 1 (Consultant editors: H. L. Kornberg, D. C. Phillips; volume editor: H. Gutfreund). Butterworths, London - University Park Press, Baltimore 1974; stron 411, cena £ 8.50.

Chemistry of Macromolecules dotyczy ostatnich osiągnięć w badaniach budowy cząsteczki białkowej. Strukturę I-rzędową omówiono interesująco w aspekcie zmian zachodzących w procesie ewolucji (J. Williams, Bristol). Budowę podjednostkową, trójwymiarową oraz konformację białka przedstawiono w trzech rozdziałach (M. A. Joynson, Bristol; H. Muirhead, Bristol; F. M. Pohl, Goettingen), zwracając uwagę na konieczność stosowania w tych badaniach — oprócz analizy rentgenowskiej i mikroskopii elektronowej — również analizy spektroskopowej oraz metod termodynamicznych, hydrodynamicznych i kinetycznych. Konformacja makrocząsteczki w kryształach jest inna niż w roztworze, a w stanie natywnym w komórce jest bardzo zależna od czynników zewnętrznych i zmienia się w procesie katalizy enzymatycznej.

W wyjaśnieniu przestrzennej budowy białka podkreślono znaczenie badań prowadzonych na naturalnych i syntetycznych polimerach, szczególnie przy zastosowaniu modyfikacji chemicznych grup funkcyjnych (J. R. Knowles, Oxford). Omówiono teoretycznie metodykę podstawiania oraz podano liczne przykłady zastosowań w badaniu budowy białka ze szczególnym uwzględnieniem metaloproteidów (J. E. Coleman, Yale). Jeden z rozdziałów poświęcono omówieniu, z podaniem podstaw teoretycznych, powszechnie stosowanych metod fizyko-chemicznych badania kształtu i wielkości cząsteczki białka (C. F. Phelps, Bristol). Wydaje się, że w artykułach dotyczących kinetyki reakcji (H. Gutfreund, Bristol) oraz regulacji aktywności enzymatycznej zależnej od modyfikacji struktury białka (C. I. Pogson, Canterbury) zbyt mało uwagi poświęcono regulacji hormonalnej. Na zakończenie podano przegląd aktualnych poglądów na temat funkcji białek w regulacji procesów komórkowych (J. R. Knowles, Oxford i H. Gutfreund, Bristol).

Chemistry of Macromolecules dotyczy głównie chemii białek, jednakże dokładne i krytyczne omówienie najnowszych metod fizyko-chemicznych czyni książkę użyteczną również w badaniach struktury polinukleotydów i polisacharydów. Książka jest bogato ilustrowana schematami i fotografiami i jest zaopatrzona w indeks rzeczowy. Każdy z dziesięciu artykułów zawiera wykaz cytowanej literatury (obejmujący pozycje tylko do 1972 r.).

Książka otwiera 12-tomową serię z zakresu biochemii i zawiera zapowiedź kontynuowania wydawnictwa na lata następne.

Teresa Laskowska-Klita

MTP International Review of Science, PLANT BIOCHEMISTRY, Biochemistry, series one vol. 11 (Consultant editors: H. L. Kornberg, D. C. Phillips; volume editor: D. H. Northcote) Butterworths, London - University Park Press, Baltimore 1974; stron 287; cena £ 8.50.

Zadaniem serii wydawniczej stanowiącej Międzynarodowy Przegląd Naukowy jest dostarczanie regularnych i autorytatywnych informacji w takich dziedzinach nauki jak biochemia, fizjologia, chemia organiczna, chemia nieorganiczna i chemia fizyczna. Seria biochemiczna obejmuje dotychczas 12 tomów, książka *Plant Biochemistry* stanowi 11 tom z tej serii. Książka zawiera siedem artykułów przeglądowych poruszających problemy, które dotychczas nie były opracowane w sposób wyczerpujący. Przegląd cytowanej literatury obejmuje pozycje do roku 1973. Tematy przedstawione w powyższym tomie są unikalne dla roślin, aczkolwiek wiele z poruszanych problemów ma charakter bardziej ogólny i znajduje odzwierciedlenie w innych gałęziach biochemii.

Rozwój technik badawczych doprowadził w ostatnich latach do powstania nowych koncepcji na temat struktury błon komórkowych. Omówienie nowych technik badawczych w odniesieniu do chloroplastów, dane dotyczące chemicznej struktury ich błon oraz modele transportu metabolitów do i z organelli są tematem rozdziału pierwszego, opracowanego przez D. A. Walkera (Sheffield). W rozdziale drugim A. Haug (Trondheim) przedstawia dane na temat struktury polisacharydów będących składnikami ściany komórkowej alg. Autor omawia metody izolacji i problemy homogenności materiału, strukturę i biosyntezę polisacharydów oraz informacje dotyczące ich roli biologicznej. Szlaki katabolizmu węglowodanów u roślin wyższych stanowią przedmiot rozdziału trzeciego (T. Rees, Cambridge). Autor rozważa metody oznaczania metabolitów przemiany węglowodanowej oraz czynności enzymów katalizujących te przemiany, dyskutując możliwości różnic między wynikami badań w warunkach *in vitro* a stosunkami w tkankach nienaruszonych. Szczegółowe dane dotyczą katabolizmu głównych zapasowych węglowodanów roślinnych, t.j. skrobi i sacharozy, oraz szlaków utleniania heksoz. Substancje wzrostowe roślin, ich występowanie, biosynteza, metabolizm, fizjologiczne znaczenie zmian stężeń tych związków oraz dotychczasowe dane na temat mechanizmu ich działania stanowią temat rozdziału czwartego (D. G. Morgan i C. B. Morgan, Cambridge). W rozdziale piątym H. Smith (Nottingham) omawia pierwotne mechanizmy odbioru bodźców świetlnych przez rośliny oraz wtórny wpływ światła na aktywność i syntezę enzymów warunkujących różnicowanie się tkanek roślinnych. Przedmiotem dwóch ostatnich rozdziałów są unikalne dla roślin systemy enzymatyczne warunkujące przyswajanie azotanów i azotynów (E. J. Hewitt, Bristol) i metabolizm pierścieni aromatycznych będących prekursorami flawonoidów i ligniny (G. N. H. Towers, British Columbia).

Rozważając poszczególne tematy autorzy i wydawcy książki zwrócili uwagę, aby dostarczone informacje przedstawić w sposób przystępny i włączyć je w kontekst wiedzy dotychczasowej, umożliwiając zrozumienie nowych poglądów przez biochemików nie będących specjalistami z danej dziedziny, jak również przez zaawansowanych studentów.

Andrzej Rafalski

KOMUNIKAT

W dniach od 26 do 28 maja 1976 r. odbędzie się w Poznaniu XIV Sympozjum Polskiego Towarzystwa Histochemików i Cytochemików z następującą tematyką:

1. Zmiany cytochemiczne i histochemiczne w zatruciach przemysłowych i rolniczych.
2. Nowe metody cytochemiczne i histochemiczne.
3. Aparat Golgiego.

Termin zgłaszania uczestnictwa i doniesień upływa z dniem 31 grudnia 1975 r.

Adres Komitetu Organizacyjnego:

Doc. dr habil. Mirosław Kozik
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